CHAPTER TWO

LITERATURE REVIEW

2.1 Phototrophic Bacteria

Phototrophic bacteria previously known as photosynthetic bacteria (PSB) were described first by Engelmann in 1883. He characterized them based on pigmentation with well defined absorption spectrum similar to green plant. Classical studies on photosynthetic bacterium (PSB) were started in 1931 and classified phototrophic bacteria as photosynthetic bacteria with the isolation of two types of PSB, one purple and the other green (Van Niel 1957). Van Niel defined four conditions for their isolation and growth as (a) illumination, (b) mineral medium, (c) a light alkaline reaction, and (d) strict anaerobiosis. He also mentioned the overall metabolic pathway as:

(a) Green bacteria $2H_2S + CO_2 \xrightarrow[\text{Energy}]{\text{Light}} (CH_2O) + H_2O + 2S$

(b) Purple bacteria, $H_2S + 2H_2O + 2CO_2 \xrightarrow[\text{Energy}]{\text{Light}} 2(CH_2O) + H_2SO_4$

Green bacteria oxidize sulfide to sulfur with the assimilation of carbon dioxide, but in purple bacteria oxidation of sulfide proceeded through sulfate (Kobayashi & Kobayashi 2001).

Dow (1982) reported three distinct groups of photosynthetic bacteria (PSB). They are cyanobacteria, purple PSB and green PSB. The major distinction is that phototrophic (with only one photosystem) bacteria can synthesize in anoxygenic condition,

whereas the cyanobacteria with two photosystems operating in series display oxygenic photosynthesis. Phototrophic bacteria do not use water as an electron donor, but cyanobacteria do so. Phototrophic bacteria unlike cyanobacteria in the presence of light can also use a variety of simple organic compounds as electron donors (Pfenning 1977; Imhoff 1992).

However, three major groups of photosynthetic bacteria are now recognized:

(i) the purple and brown non-sulfur bacteria (Athiorhodaceae)
(ii) the purple sulfur bacteria (Thiorhodaceae), and
(iii) the green sulfur bacteria (Chlorobacteriaceae)

Phototrophic bacteria are widely distributed in nature, predominantly aquatic and terrestrial habitats and even under the extreme conditions of Antarctica (Herbert 1976; Madigan 1999). In aquatic habitats phototrophic bacteria can be found in freshwater, estuarine water and seawater, sulfur containing hot springs (Pfenning 1967; Imhoff 1992) and in agro-industrial wastewater (Sasaki et al. 1991; Prasertsan et al. 1993).

The facultative anaerobic phototrophic bacterium *Rhodobacter sulfidophilus* was first isolated from marine mud in Netherlands (Hansen and Veldkamp in 1973). The unique property of this bacterium is its the ability to utilize hydrogen sulfide and its remarkable high sulfide tolerance.

Rhodobacter species share some of the common physical and biochemical characteristics. Pair-wise sequence comparisons and distance matrix analysis, however, showed two major clusters. One cluster included only freshwater and terrestrial species, and the other cluster contained marine species (Haraishi & Ueda 1994). Phenotypically, marine *Rhodobacter* species can be differentiated based on salt requirement for optimal growth, sulfide tolerance, final sulfide oxidation product, and

polar lipid composition. Haraishi & Ueda (1994) has suggested all marine *Rhodobacter* spp. should be moved to new genus *Rhodovulum* gen. nov. (*Rho do vu lum*. Gr. N. *rhodos*, rose; L. dim. n. *ovulum*, small egg; M.L. neut.n. *Rhodovulum*, small red egg). *Rhodovulum sulfidophilum* comb. nov. is the type species. They also mentioned that *Rhodovulum* requires salt for normal growth. *Rhodovulum* has a higher tolerance for sulfide compared to *Rhodobacter* spp. and the final oxidation product of *Rhodovulum* is sulfate. In *Rhodobacter* spp. the final product is elemental sulfur. The *Rhodovulum* spp. contain sulfolipids (probably sulfoquinovosyldiglyceride) but not phosphatidylcholine. Nucleotide sequence based on 16S rRNA, could also be used to differentiate the genera *Rhodovulum* and *Rhodobacter* sp. (Haraishi and Ueda 1994).

2.2 Purple Non-sulfur Phototrophic Bacteria

The purple phototrophic bacteria are classified in to two broad distinct groups, that is, the purple non-sulfur bacteria (PNSB) and purple sulfur bacteria (PSB). The purple non-sulfur bacteria belong to Athiorhodaceae and are photoheterotrophic, using organic compounds as both electron donors and carbon sources. This group of bacteria can reduce CO_2 but derive most of their cellular material from organic nutrients. The specialty of purple non-sulfur bacteria is their ability to grow both under anaerobic light and aerobic dark conditions. Most of the research that have been carried out with purple non-sulfur photosynthetic bacteria are for the benefits of human kind. Purple non-sulfur bacteria are widely utilized, because of their wide distribution in nature, ability to grow in a variety of substrates and ability to grow in different culture conditions.

Nishizawa et al. (1974) stated that PNSB strain *Rhodopseudomonas sphaeroides* can grow both under aerobic and anaerobic conditions under dark and light systems, using

glucose and lower fatty acids as single or in a mixture of carbon sources. In aerobic dark condition, specific growth rate (SGR) was two times higher than in anaerobic light, but cell yield was lower. They also cited Hirayama et al. (1974) had reported that biosynthesis of carotenoid in *Rhodobacter capsulatus* was repressed by dissolved oxygen under aerobic light conditions.

Rhodopseudomonas capsulata is one of the interesting species capable of growing in light and dark modes (Madigan et al. 1980). The species can grow anaerobically in darkness in synthetic media with glucose as the sole energy and carbon source, provided that DMSO (dimethyl sulfoxide) was added. DMSO was reduced to dimethyl sulfide during growth, but the energy conversion process supporting growth was not defined (Madigan et al. 1980). They also reported that *R. capsulata* can grow under anaerobic dark condition with fructose as the carbon and energy source and trimethyl amine N-oxide (TMAO) as the accessory oxidant. TMAO, is the most common organic compound produced by various marine animals. Madigan et al. (1980) concluded that DMSO and TMAO containing compounds might play a significant ecological role by facilitating the photosynthetic bacteria to grow under dark fermentative condition.

The ability of various anoxygenic phototropic bacteria to grow in absence of light has been established and a variety of physiological mechanisms have been implicated (Madigan 1988). The bacteria develop mechanisms for obtaining growth energy through alternative sources. He also pointed that purple non-sulfur bacteria are the most versatile in this regard, being capable of either respiratory or fermentative growth under appropriate culture conditions. On the other hand, Kragil'nikora et al. (1983) observed that purple sulfur bacteria showed more restricted growth in dark

fermentation, but chemo-organotrophic and lithotrophic dark growth have been reported. Several purple non-sulfur bacteria are also able to grow chemo-lithotrophically in the dark, where hydrogen serves as an energy source. Oxygen is used as the terminal electron acceptor and carbon dioxide as the sole carbon source (Madigan & Gest 1979).

Phototrophic bacterium strain *Rhodobacter sphaeroides* could be cultivated in synthetic media under aerobic-dark, microaerobic-light and anaerobic-light conditions (Sasaki et al. 1991). In aerobic-dark and microaerobic-light cultures, growth and reduction of COD were remarkably different compared to culture in anaerobic-light condition. Volatile fatty acids were quickly consumed when cultured in the fermenter. In microaerobic-light culture, the maximum cell biomass of 3.7 g/L, was relatively higher compared to biomass of 2.0 g/L in aerobic-dark culture. Growth rate in micoaerobic-light culture was relatively slow because of limitation of light illumination (Sasaki et al. 1991).

The PNBS characterized for growth in agro-based wastes and wastewaters had different growth parameters as compared to that in synthetic medium. Different factors like light intensity, types of lamps, temperature and pH had significant effects on the growth and biomass production. Prasertsan et al. (1993) isolated the purple non-sulfur bacterium *Rhodocyclus gelatinosus*, from seafood processing wastewater. The bacterium gave higher specific growth rate (SGR) and cell yield under aerobic light and dark culture conditions when cultured in wastewater. Purple non-sulfur bacteria are facultative anaerobes, heterotrophs and able to oxidize compounds photosynthetically or in the dark aerobically. Cell pigments production, however,

could be the highest under anaerobic-light conditions compared to aerobic condition (Madigan 1988).

Comparative studies on the efficiency of different light intensities under 1000, 3000 and 5000 lux in anaerobic culture condition showed that the highest cell mass of 5.6 g/L (w/w) with 50% cell protein and a COD removal of 86% were obtained in 3000 lux (Prasertsen et al. 1993). On the other hand, 5000 lux intensity resulted in an increased temperature (38 ^{0}C), higher than the optimal temperature for the growth of many of the strains of *R. gelatinosus* (Prasertsen et al. 1993).

Not only the light intensity, but the type of lamp used also influenced the biomass production. Prasertsen et al. (1993) observed higher specific growth rate (0.03 per h), biomass concentration (5.6 g/L), cell yield (0.33g cells/g COD), carotenoid production (1.31mg/g dry cell weight), bacteriocholorophyll contains (11.67 mg/g) and protein contains (44%) using tungsten lamp as compared to fluorescent lamp. Sawada et al. (1977) reported that tungsten lamp generated light in the 800 to 900nm range, which was required for the growth of PSB. Prasertsen et al. (1993) also reported that cell growth, carotenoid pigments and bacteriochlorophyll developed well within a range of 6.0 to 7.5 pH, but were the highest at neutral pH-7. This optimal pH was similar to those reported for other photosynthetic species (Pfenning 1967, Sasaki & Nagai 1979). Changes in pH of various media during phototrophic growth were normally associated with the assimilation or release of CO_2, the production of H_2SO_4 by the oxidation of H_2S or thiosulfate and by the accumulation of organic acids in the medium. In some cases, the pH changes in the medium indicated the utilization organic acids.

Qualitative and quantitative compositions of carotenoid depend on the culture media, culture techniques, oxygen saturation and light intensity. Purple non-sulfur bacteria growing in organic substrates in the dark utilize a purely respiratory metabolism, using the Kreb's cycle as a pathway for terminal substrate oxidation (Shipman et al. 1977). Oxygen remarkably influences carotenoid synthesis by photosynthetic bacterial cells. Caroteniod synthesis by photosynthetic cells cultivated both in light and dark conditions is almost totally suppressed by vigorous aeration. Similarly, exposure to light also lead to changes in the quantity of cellular carotenoid content, as light intensity increases carotenoid synthesis decreases. The exact mechanism by which photosynthetic bacteria controlling pigments biosynthesis is unknown. When growing aerobically in the dark on organic compounds, pigment synthesis is repressed so that energy made available by oxidative reactions not diverted into unnecessary materials (Shipman et al. 1977).

There is hardly any literature on the potential of *Rhodobacter sulfidophilus*, except for sulfate metabolism studies (Shioi & Doi 1990). This bacterium could grow and synthesize bacteriochlorophyll-a aerobically both under the dark and light conditions. Furthermore, the bacterium was capable of photosynthesis under both anaerobic and aerobic light conditions. Thus, it was concluded that *Rb. sulfidophilus* is a link between the facultative anaerobic and aerobic photosynthetic bacteria. Comparative growth and bacteriochlorophyll formation of different species under various conditions are shown in the Table 2.1 and Table 2.2.

Purple non-sulfur phototrophic bacteria (PNSB) are widely studied and used for a number of commercial purposes (Sasikala & Ramana 1995). These bacteria are the most diverse and useful group among all anoxygenic PSB. They are most widely

distributed and can easily be enriched and grown. These bacteria are preferably grown by a photo-organic metabolism with simple organic substrates. However, most species are capable of growing photolithoautotrophically with molecular H_2 as electron donor. Although PSB have been known for almost two centuries, their potential for various biotechnological applications has only recently been recognized (Burgess et al. 1991; Burgess et al. 1993)

Table 2.1 Comparative growth and bacteriochlorophyll (Bchl) formation in three strains of phototrophic bacteria under different culture systems (Shioi & Doi 1990)

Growth conditions	*R. sulfidophilus*		*R. sphaeroides*		*Erythrobacter* sp.	
	Growth	Bchl	Growth	Bchl	Growth	Bchl
Anaerobic light	1.61	15.0	1.88	15.6	0.084	0.23
Anaerobic dark	0.14	0.42	0.14	0.55	0.078	0.23
Semianaerobic light	1.73	14.2	1.60	12.1	0.26	0.27
Semianaerobic dark	0.21	0.61	0.14	0.44	0.23	0.51
Aerobic light	1.91	1.52	1.14	0.35	1.28	0.56
Aerobic dark	1.80	11.1	1.09	0.89	1.23	4.63

Growth: mg protein/mL; Bachl: nmol/mL; culture conditions: dark at 6000 lux at 28 ^{0}C for 24 h.

Table 2.2 Effect of light on the aerobic growth of *Rhodobacter sulfidophilus* (Shioi & Doi 1990)

Growth conditions	Biomass (mg/mL)	% of biomass	Bchl (nmol/mL)	% of Bchl.
36 h dark	2.43	100	12.38	100
36 h light	2.59	106	1.60	13
12 h dark+24 h light	3.28	135	5.96	48

Rhodobacter sulfidophilus were grown aerobically in the dark or light (6000 lux) at 28 ^{0}C for 36 h.

Watanabe et al. (1998) reported that *Rhodovulum* sp. grow well under aerobic dark culture condition. Further, Sakato et al. (1992) has developed aerobic dark cultures

and reported high cell density culture are impossible under anaerobic condition with illumination because of limited light supply.

2.2.1 Nutritive Values

Phototrophic bacteria are being utilized for various purposes because of thire good nutritional profiles. The bacteria contained protein, lipids, vitamins and minerals, carotenoids and other co-factors, which could have potential applications in different areas. The nutritional profiles depend on the culture conditions, strains and substrates used. When cultured in different industrial liquid wastes, the biomass contained 40-69% protein, 0.09-0.80 mg carotenoid/gm dry cell, 30-79 mg vitamin B_{12}/kg dry cell weight and essential amino acids and this composition was comparable to egg, algae and soybean meal (Kobayashi & Kurata 1978).

Photosynthetic bacterial biomass is not only rich in high quality protein, but also contained significantly large amount of carotenoid pigments, biological co-factor and vitamins (Kobayashi & Kurata 1978; Vrati & Verma 1983). In addition the biomass found to be rich in enzymes and active compounds. Phototrophs grown in miso factory wastewater under aerobic dark condition contains 63% (w/w) protein, 23 μg vitamin B_{12} and 24 μg carotenoid/g dry cell weight (Sasaki et al. 1991). On the other hand, cell pigmentation depends on the culture conditions (Prasertsan et al. 1993). Cell pigments were the highest under anaerobic light condition when compared with aerobic (Prasertsan et al. 1993) (Table 2.3).

Table: 2.3 Cell pigments at different culture systems

(mg/g dry cell weight)	Anaerobic	Aerobic
Carotenoid	11.8 – 12.6	1.5 – 2.1
Bacteriochlorophyll	102.0 – 108.0	11.7 – 15.9

Cohen-Bazire et al. (1957 as cited in Noparatnaraporn & Nagai 1986) reported that there was a lower pigment formation under aerobic condition as because oxygen inhibited the pigment synthesis and acted as a bleaching agent. The red and yellow carotenoid pigments formed under static anaerobic light conditions were identified as compounds of spheroidenone and spheroidene respectively.

Suppression of pigment accumulation by light is commonly observed in aerobically grown cells of *Rhodobacter sulfidophilus* (Shioi & Doi 1990). The peak values of different compounds in terms of spectrum distribution are shown in Tables 2.4, 2.5 and 2.6.

Table 2.4 Types of carotenoid and their peak of absorbance in living cells (Pfennings & Truper 1991)

Major carotenoid compounds	Absorbance maxima (nm)
Lycopene and rhodopin	463, 490, 524
Spirilloxanthin	486, 515, 552
Spheroidene	450, 482, 514
Rhodopinal	497, 529
Okenone	521

Table 2.5 Major carotenoid groups of anoxygenic phototrophic bacteria (Schmidt 1978)

Biosynthetic groups	Major compounds
Normal spirilloxanthin series	Lycopene, Rhodopin, Spirilloxanthin
Rhodopinal series	Lycopene, Lycopenal, Lycopenol, Rhodopin, Rhodopinal, Rhodophenol
Alternative spirilloxanthin series	Hydroxyneurosporene, Sphaeroidene, Sphaeroideneone (Spirilloxanthin)
Okenone series	Okenone
Isorenieratene series	B-Carotene, Isorenieratene
Chlorobactene series	Γ-Carotene, Chlorobactene

Table 2.6 Characteristics of maximum absorbance values of bacteriochlorophylls in bacterial living cells (Dow 1982; Imhoff 1995)

Bacteriochlorophyll	Absorption maxima (nm)
A	375, 590, 800-810, 830-890
B	400, 605, 835-850, 1015-1035
C	335, 460, 745-760, 812
D	325, 450, 725-745, 805
E	345, 450-460, 715-725
G	370, 419, 575, 670, 780-790

Shioi & Doi (1990) concluded that this bacterium contained Bch1- protein complexes, which absorbed light at 802nm, 860nm and around 885nm in the near infrared region. Carotenoid compositions were also quite different between anaerobically and aerobically grown cells. High and low oxygen saturation limited carotenoids synthesis, but synthesis could be maximized with optimal oxygen saturation.

The nutritive values of phototrophic bacteria were comparable with other conventional feeds used in livestock, fish and poultry. Normally, waste grown bacterial biomass have certain limitations, but these are insignificant when compared with other feeds. Shipman et al. (1977) compared the essential amino acids (EAA) of phototrophic bacteria with that of egg protein and soy protein (Table 2.7). The proximate composition, vitamins and minerals in phototrophic bacteria studied by different authors are shown in Table 2.8 2.9 and 2.10. Getha et al. (1998) compared the proximate composition of *Rhodopseudomonas palustris* grown in sago starch processing wastewater and in synthetic medium. The essential amino acid content varied with culture substrates used (Table 2.11).

Table 2.7 Comparison of Essential Amino Acid (EAA) in photosynthetic bacteria with conventional feed(Shipman et al. 1977)

Amino acid	Egg protein (%)	Soya protein (%)	Photosynthetic bacterial protein (%)
Histidine	2.4	2.4	3.4-3.9
Isoleucine	6.6	5.4	4.1-4.3
Leucine	8.8	7.7	7.4-7.9
Lysine	6.4	6.3	5.6-6.0
Methionine	3.1	1.3	3.0
Phenylalanine	5.8	4.3	4.3-4.6
Threonine	5.0	3.9	2.9-4.4
Tryptophan	1.6	1.4	-
Valine	7.4	5.2	6.5-7.0

Table 2.8 Comparison of vitamins in phototrophic bacteria and yeast (Shipman et al. 1977)

Vitamin	Photosynthetic bacteria (μg/100 gm dried cells)	Yeast[a] (μg/100gm dried cells)
Riboflavin (B_2)	3600	2900
Pyridoxine (B_6)	3000	2400
Folic acid	2000	1700
Vitamin B_{12}	200	10
Ascorbic acid (C)	20000	-
Cholecalciferol (D_3)	10000	300000[b]

[a]Adapted From Kolayashi (1970).

[b]International Units (I.U.)

Table 2.9 Proximate composition (%) of different species of phototrophic bacteria grown in agro based industrial wastes (Sasaki et al. 1991).

	Crude protein	Crude fat	Carbohydrate	Crude fiber	Ash
Rhodocyclus gelatinosus[a]	56	2.45	26.4	-	3.21
Rhodobacter sphaeroides[b]	66.6	1.88	24.9	2.95	3.62

[a]Growth on cassava starch medium (aerobic dark culture)

[b]Growth on pineapple waste (aerobic-dark culture)

Table 2.10 Contents of vitamin in different strains of Purple Non-sulfur Bacteria (PNSB) bacterial cells (Sasaki et al. 1991)

Name of Vitamins	*Rhodocyclus gelatinous*	*Rhodobacter sphaeroides*	*Rhodobacter capsulatus*[b]	Yeast[c]
B_1	na	na	12	11-13
B_2	33.2	13.0	50	110-130
B_6	na	na	5	4.8-7.6
B_{12}	33	78	21	trace
E	51	210	na	na
Carotenoid	90	800	na	na
Nicotinic acid	136	58	125	165-200
Folic acid	7.2	1.0	60	1.8-2.4
Panthothenic acid	na	na	30	14-23
Biotin	8.3	6.3	65	110-130

[b]Kobayashi & Kurata (1978); na = not available
[c]Adapted From Kobayashi (1970).

Table 2.11 Comparison of proximate compositions and essential amino acids in *Rhodopesudomonas palustris* grown in sago effluent and in synthetic media (Getha et al. 1998).

	Synthetic media	Sago wastewater
Crude protein (%)	56.2	40
Crude lipid (%)	2.78	0.64
Carbohydrates (%)	25.0	not determined
True protein (%)	55	31
Amino acid (% dry weight)		
Lysine	2.60	1.88
Histidine	1.02	0.70
Threonine	2.38	1.47
Valine	3.21	2.02
Methionine	1.26	0.58
Isoleucine	2.21	1.42
Leucine	5.16	2.82
Phenylalanine	5.90	2.51
Arginine	3.93	2.24
Tryptophan	1.04	0.71

Phototrophic bacteria lack highly unsaturated and poly-unsaturated fatty acids. The fatty acids profiles in most of the purple non-sulfur bacteria are similar. The bacteria have certain common characteristics in their membranes and thus variability in lipid compositions and in fatty acids is minimal (Imhoff & Imhoff 1995).

Purple non-sulfur phototrophic bacteria also show great diversity in habitat and growth under different conditions and thus the polar lipid compositions. Some *Rhodobacter* species contain significant amounts of plant type sulfo-lipids. The proportions of sulfo-lipids in *Rhodobacter sulfidophilus* are observed to be significantly influenced by the sulfur sources. Sulfate and reduced sulfur compounds e.g. thiosulfate and cysteine served as sulfur sources for sulfolipids in *R. sulfidophilus* sp. (Imhoff & Imhoff 1995). The lipid components of phototrophic bactria are shown in Table 2.12.

Imhoff & Imhoff (1995) reported that phototrophic purple non-sulfur bacteria generally contained saturated and mono-unsaturated straight chain C-16 and C-17 fatty acids (Table 2.13). All fatty acids were changed or modified during growth due to different growth conditions and substrates. These were the factors responsible for greater variations in fatty acid compositions as reported in different studies.

Table 2.12 Presence of some major polar lipids in *Rhodobacter*[a] species (Imhoff & Imhoff 1995)

	PG	CL	PE	PC	SQDC	OA	APL	PL1	PL2
Rb. sphaeroides	+	+	+	+	+	+	-	+	+
Rb. capsulatus	+	-	+	+	-	+	-	+	-
Rb. veldkampii	+	-	+	-	-	+	+	-	-
Rb. sulfidophilus	+	-	+	-	+	+	-	+	-
Rb. adriaticus	+	-	-	-	+	+	-	-	-
Rb. euryhalimus	+	+	+	-	+	+	-	-	-

"+" stands for present of compounds and "-" means compound absent

PG- Phosphatidylglycerol, CL- Cardilipin, PE- Phosphatidylethanolamine, PC- Phosphatidylcholine, SQDC- Sulfoquinovosyldiglyceride, OA- Ornithine amide, APL- Aminophospholipid, PL1- Phospholipid.

Polar lipids and fatty acids composition are completely dependent on different growth condition (Imhoff & Imhoff 1995). However, few studies has been conducted on the

effects of different growth conditions and environment on the composition of fatty acids and lipids. The proportion of sulfolipid in *Rhodobacter sphaeroides* increases under phosphate limiting growth condition (Imhoff & imhoff 1995).

Table 2.13 Major fatty acid composition in *Rhodobacter* species (Imhoff & Imhoff 1995)

Species	Strains	14:0	16:0	16:1	18:0	18:1	References
Rb. sphaeroides(5)*	2	0.2 –0.3	3.9-8.3	1.2-2.0	1.9-16.2	72.0-77.9	Imhoff 1991
	GA	-	1.7	1.0	4.8	90.8	Wood et al. 1965
		tr	5.5	1.6	12	80	Marinett & Cattieu 1981
		-	7.8	1.5	10.7	75.9	Urakami & Komagata 1988
Rb. capsulatus(6)*	17023	0.1-0.4	4.1-5.0	5.1-7.4	3.8-9.3	78.1-84.2	Inhoff 1991
		-	2.3	2.4	4.2	90.3	Wood et al. 1965
		tr	1.9	2.6	1.3	93.6	Schroder et al. 1969
		-	13.8	15.0	4.0	63.5	Urakami and Komagata 1988
Rb. veldkampii(1)*	2,3,11	0.1	4.3	17.5	6.5	69.4	Imhoff 1991
Rb. adriaticus(4)*	3764	0.2	3.8-6.4	0.2-0.4	19.8-22.0	61.6-67.2	Imhoff 1991
Rb. sulfidophilus(4*)	NCIB824	0.1-0.3	11.6-14.6	0.7-1.5		72.8-75.0	Imhoff 1991
			16.0	0.4	7.6-9.8	72.1	Urakami and
			17.8	3.0	2.4	72.2	Komagata 1988
	DSM1374				4.0		
Rb. euryhalimus(1)*	DSM2351	0.1	4.9	2.5		67.5	Imhoff 1991
					21.8		

Substrate or nutrient medium containing lipid components or precursor molecules showed significant effect on the incorporation of lipid and fatty acids into the bacterial membrane (Imhoff & Imhoff 1995). An increase in temperature caused a decrease of C-18 and unsaturated fatty acids in certain phototrophic bacteria, while C-16 and saturated fatty acids were increased. These changing effects were due to mechanisms responsible for regulating membrane fluidity and bi-layer stability of the membrane in response to temperature changes (Imhoff & Imhoff 1995).

1988). The cellular contents of saturated fatty acids were found to be lower in chemotrophically grown cells of *Rhodopseudomonas palustris*, *Rubrivivax gelatinosus* and *Rhodospirillum rubrum* (Hands & Bartley 1962, cited by Imhoff & Imhoff 1995).

Besides good nutritional profile the phototrophic bacteria also possess certain other co-factors (Shipman et al. 1977). Phototrophic bacteria have been screened for anti-viral substances outside the cells, which contributed to the suppression of viral spread in nature (Kobayashi & Kondo 1984). They observed that phototrophic bacteria *Rhodobacter capsulata* contained antiviral substances and inactivate Sindbis virus. Various antiviral activities have also been examined using purified preparations. They also mentioned that photrophic bacterium *Rhodobacter capsulata* could inactivate pathogenic viruses, such as polio, influenza, mumps and herpes simplex. Furthermore, these anti viral substances are not found to be inhibitory to normal animal cell metabolism.

2.2.2 Phototrophic bacteria in waste water utilization

One of the most useful and cheap substrate for phototrophic bacteria to grow in is organic wastewater, which also lead to the bioremediation of the wastewater.

Kobayashi et al. (1978) utilized phototrophs for the purification of polluted industrial wastewaters. Heavily polluted industrial wastewater could also be purified with the addition of phototrophic bacteria (Table 2.14 and Table 2.15).

Table 2.14 Treatment of original waste solution with microbial production (Kobayashi & Tchan 1973)

Types of waste solutions	BOD values in ppm		Yields of photosynthetic bacterial cells (g/L)
	Original	After Treatment	
Starch industry	>10,000	600 – 1000	1.0 – 5.0
Wool washing factory	5000 – 10,000	200 – 500	1.0 – 3.0
Canned food factory (Fish meat)	200 – 5000	100 – 400	0.5 – 1.0
Pharmaceutical fermentation Industry (penicillin, erythromycin)	2000 - 5000	500 – 800	0.8 – 3.0

Table 2.15 Treatment of polluted industrial waste containing dense organic substances (Kobayashi et al. 1978)

Parameter	Original	Supernatant after PSB treatment	Purified Effluent
BOD (ppm)	6600	380	15
COD (ppm)	3364	354	64
Suspended solid (ppm)	6540	450	17
Kjeldhal Nitrogen (ppm)	915	32.8	7.8
pH	6.8	7.3	7.1

Most of the phototrophic bacteria have been observed to be involved in reducing COD of industrial wastewater. Phototrophic bacteria are used to treat industrial wastewater and in particular agricultural wastewaters with simultaneous production of single cell protein. Gaighet et al. (1982) observed that photosynthetic bacteria could utilize fermented vegetables canning factory effluent as a nutrient source and reduced the organic load. A 94% COD reduction was observed in the photosynthetic bacterial culture unit and a 98% in second step where a mixed culture of *Chlorella* and *Scenedesmus* was used to treat the waste. Algae grew well in series with photosynthetic bacteria, but unknown factors caused growth inhibitions, which were not mentioned in the studies.

Noparatnaraporn et al. (1986) uses a variety of agro-industrial wastes as substrates for the culture of *Rhodobacter sphaeroides* strain P47 as a source of SCP. The strain was also used to treat pineapple waste too. Phototrophic bacteria that have been used in the utilizations of industrial waste are summarized in Table 2.16.

Table 2.16 Sources of waste material and wastewater purified by phototrophic bacteria (Source: Getha 1996)

INDUSTRY	WASTE MATERIAL	PNB SPECIES	REFERENCES
Microbial (fermentation industry)	Antibiotic factory effluent	Rcap	Sawada et al. (1977)
	Beer factory effluent	PTB	Kobayashi (1982)
Chemical industry	Textiles, synthetic fibres, synthetic Resins effluent	PTB	Kobayahi (1982)
	Wool scour liquor	Rcap	Sawada et al. (1977)
	Chemical fertilizer effluent	PTB	Kobayashi et al. (1982)
	Aromatic organic compounds Containing effluent	Rpal	Harwood & Gibson (1988) Rahakar et al. (1993)
	Heavy metal containing effluent	Rspr	Vatsala (1987)
	Chlorinated hydrocarbons containing	Rsph	Moore & Rowell (1992)
	Effluent	Rsph	Kerby and Rowell (1992)
Food industry	Fish canning effluent	PTB	Kobayashi et al. (1978)
	Tuna condensate	Rgel	Prasertsan et al. (1994)
	Soybean cake factory effluent	Rpse	Kobayashi & Ye (1986)
	Soybean cooked (miso) drain	Rgel	Sasaki et al. (1981)
	Wheat bran slurry	Rgel	Shipmam et al. (1975)
	Pineapple cannery waste	Rsph P47	Noparatnaraporn et al. (1986)
	Mandarin orange peel	Rsph	Sasaki et al. (1991)
	Sardine processing effluent	Rvsul	Azad et al. (2003)
Starch industry	Cassava sun dried solid waste	Rgel,	Noparatnaraporn et al. (1987)
	Cassava factory effluent	Rsph	Sasaki et al. (1991)
	Sago factory effluent	Rpal	Getha et.al. (1998)
Sewage & Animal wastes	Sewage treatment plant	Rcap, Rpal,	Siefert et al. (1978) Kobayashi (1982)
	Swine waste	Rgel, Rsph	Kobayashi et al. (1978) Sasaki et al. (1991)
	Anaerobic-digested cow dung	Rsph Rcap	Vrati & Verma (1983)
Others	Ammonia-treated straw-whey silage	Rcap	Suhaimi et al. (1988)
	Sugar refinery wastes	Ral	Vincenzini et al. (1982)
	Straw paper mill effluent	Rpal	Vicenzini et al. (1982)
	Palm oil mill effluent (POME)	Rsph	Hassan et al. (1994)

PTB Phototrophic bacteria; PSB V1 Phototrophic bacteria strain V1;
Rpse *Rhodopseudomonas* sp; Rpal *Rhodopseudomonas palustris*;
Rcap *Rhodobacter capsulatus*; Rgel *Rubrivivax gelatinosus*;
Rsph *Rhodobacter sphaeroides* Rsph P47 *sphaeroides* strain P47;
Rvsul *Rhodovulum sulfidophilum* Rspr *Rhodospirillum* sp.

Rhodocyclus gelatinosus was successfully grown in soybean curd and miso (fermented soybean) wastewater (Sasaki et al. 1991). This bacterium reduced COD from 30.5 to 20.5 g/L in which 73% of total sugar and 59% of soluble protein was consumed after a 40 h culture. Similarly, *Rhodocyclus gelatinosus* was also capable of utilized cassava starch directly (Noparatnaraporn et al. 1987). Prasertsan et al. (1993) reported that *Rhodocyclus gelatinosus* isolated from wastewater of seafood processing plants was able to use organic matter in the wastewater. The characteristics of seafood processing wastewater are shown in Table 2.17.They observed that dilution of tuna condensate 1:10 (v/v) with shrimp blanching was the best substrate for *Rhodocyclus gelatinosus* growth. Phototrophic bacteria grown in wastewater could have a two folds benefit: single cell protein could be produced and the cost of wastewater treatment could be reduced.

Table 2.17 Characteristics of wastewater from the seafood processing plant (Prasertsan et al. 1993)

Parameter	Source of effluent		
	Tuna condensate	Shrimp-blanching water	Frozen-seafood plant
pH	6.05	5.30	8.30
Composition (mg/L)			
COD	157080 ±500	5950 ± 530	6607 ± 475
Total nitrogen	7616 ± 124	652 ± 369	524 ± 64
Phosphate	378 ± 32	278 ± 1.5	260 ± 1
Chloride	7100 ± 0.09	8200 ± 2.00	4400 ± 2.57
Magnesium	004 ± 0.01	0.78 ± 0.05	0.29 ± 0.05
Iron	313 ± 012	0.98 ± 0.07	1.66 ± 0.79
Total solids	82218 ± 775	19.300 ± 42	23.226 ± 13
Suspended solids	6687 ± 124	984 ± 34	311 ± 21
Oil and grease	32182 ± 11	7641 ± 388	666 ± 165

Phototrophic bacteria absorbed, degraded and eliminated substances that were responsible for offal odors (Lee & Kobayashi 1992). They reported that acetic, propionic, butyric, iso-butyric, valeric and iso-valeric acids, which caused offal odor

was common in pig wastes. *Rhodobacter capsulatus* could rapidly eliminate offal odor from pig wastes.

2.2.3 Value added products from phototrophic bacteria

Phototrophs are not only used to reduce the pollution load in industrial wastewaters, but also to convert the waste nutrients into the value-added products (Table 2.18). Sasikala & Ramana (1995) investigated the production of ubiquinone, α-aminolevulinic acid (ALA) and poly– β hydroxy butyrate (PHB) using phototrophic bacteria. Tsygankov et al. (1998) had reported that purple non- sulfur bacteria *Rhodospirillum fulvum* and *Rhodobacter sphaeroides* were efficient in H_2 production and could also be used to treat wastewater from milk processing plants. Aminolevulinic acid (ALA) produced from *Rhodobacter sphaeroides* was expensive and was unstable for biochemical uses, but was found stable under acidic conditions (Sasaki et al. 2002). They further reported the possible applications of ALA as a herbicide accelerator, insecticide, medical treatment for cancer, growth promoting factors for plants, color intensifying agents for apples and increasing salt tolerance.

2.2.4 Single cell protein

Shipman et al. (1975) used marine phototrophs, to produce single cell protein (SCP) from agricultural by-products. They mentioned that PSB were not only rich in vitamins, but contained significantly large amount of carotenoids, a biological co-factor used in

Table 2.18 Value-added byproducts produced by phototrophs from municipal and agro industrial waste (Source Sasikala & Ramana 1995)

BY-PRODUCTS	References
Biomass :	
Feed supplement	Kobayashi et al. (1978)
Bio-fertilizer	Kobayashi (1982)
Chemicals:	
Bacteriocins	Guest (1974)
Vitamin B_{12}	Nishizawa et al. (1974) Sasaki et al. (1990) Sasaki et al. (1990)
Ubiquinone Q_{10}	Kobayashi & Kondo (1984)
Amylase	Buranakar et al. (1985)
Antiviral substances	Kobayashi & Hirotani (1987); Hirotani et al. (1990)
5-aminolevulinic acid (ALA)	Sasaki et al. (1989), Noparatnarporn et al. (2000)
Carotenoids	Nelis & Leenheer (1989)
Bio-plastics (PHB)	Liebergesell et al. (1991)
Antibiotic	Burgess et al. (1991)
Growth hormone	Burgess et al. (1993)
Others:	
Augmentation of biogas	Vatsala & Balaji (1987)
Hydrogen gas	Zurrer & Bachofen (1979) Thangaraj & Kulandaivelu (1994)

livestock and fish feed. Purple purple non-sulfur bacteria, such as *Rhodobacter sphaeroides*, *Rhodobacter capsulatus* and *Rhodocyclus gelatinosus* were widely used as sources of SCP, while *Rhodopseudomonas acidophila*; *Rhodopseudomonas palustris*, *Rhodospirillum rubrum* and *Rhodocyclus tenue* found to be less suitable as SCP (Sasikala & Ramana 1995).

The advantages of using phototrophic bacteria as SCP sources are (1) they could be grown in a wide range of raw carbon substrate or agro industrial wastes; (2) they have very short generation time; (3) continuous production is possible in a limited space and (4) their nutritional composition can be controlled, to certain extent by genetic manipulations.

Kobayashi & Kurata (1978) clearly mentioned that from the economic point of view utilization of single cell protein of phototrophs have several advantages as (1) waste raw materials are available free of cost; (2) can purify waste, so requires less cost for installation of waste treatment plants; (3) contamination by other microorganisms can be controlled; (4) high protein content, good amino acids profile and rich in vitamins; (5) the cells have softer cell wall and can be digested easily; (6) the bacteria are not pathogenic and are non-toxic and (7) can save energy as they can assimilate CO_2 and fix nitrogen.

Shipman et al. (1977) produced phototrophic SCP, using wheat bran as substrate and proposed a conceptual design. They also suggested the possible utilization of the SCP for human food supplementation. They concluded that hydrocarbon-based SCP process might contain toxic substances. Wheat bran, however, offered advantages of being a renewable resource and thus a toxin free, natural substrate for the production of edible protein. Other potential substrates for the production of phototrophic bacteria SCP are sewage, animal manure, and feedlot wastes. Biomass produced in sewage and animal waste could be used as supplement for various livestock (Sing & Anthony 1968, cited by Shipman et al. 1977).

Kaushik & Luquet (1980) suggested that among the non-conventional protein sources, SCP of bacterial origin appeared to be a promising substitute for fishmeal. SCP of

methanophilic bacteria fed in rainbow trout diets proved to be a substitute of fishmeal. Nearly 80% of fishmeal could be substituted with SCP without adverse effects on overall performance. Furthermore, the addition of high levels of SCP might adversely affects feed intake.

Vrati (1984) mentioned that the high production cost in SCP could limit the feasibility of PSB culture. To cut down the cost, the domestic and industrial waste, which are cheap and readily available should be used. He conducted an experiment with seven different phototrophic bacteria in the secondary treatment of cow dung clarified effluent slurry. The slurry was low in fatty acid and supported the growth of all the seven isolated phototrophic bacteria. He summarized the cell yield, percent protein and amino acid composition from seven isolates (Table 2.19 & 2.20). He concluded that *Rhodopseudomonas capsulata* biomass was a potential strain to be used as SCP.

Table 2.19 Cell yield (g/L) and crude protein contents of biomass in seven isolated phototrophic bacteria. (Vrati 1984)

Organism	Cell yield (g/L)	% Crude protein (N× 6.25)	% True protein (Lowry's)
Rps. capsulata	4.56	69.37	61.25
Rps. palustris	4.04	68.12	60.00
Rps. sphaeroides	3.02	63.12	57.50
Rps. gelatinosa	3.24	55.62	50.00
Rps. acidophila	2.75	56.87	51.25
Rps. rubrum	4.16	51.25	46.25
Rsp. tenue	4.06	48.75	40.00

Cells were harvested after 6 days of incubation at 37^{0}C under illumination.

Conversely, single cell protein grown in non-conventional substrates such as sewage, swine and antibiotic factory effluent might have problems including the accumulation of potentially harmful constituents from the substrates (Murray & Marchant 1986). They further added that mixed SCP might be useful as a protein source supplemental

at low to intermediate inclusion levels, but not recommended to be used as a major protein source. The incorporation of SCP into practical fish diets had attracted considerable interest. (Davies & Wareham 1988). Most of these SCP were derived from yeast and microbial fermentation products based on raw hydrocarbon substrates or organic waste products. In most attempts to replace high quality fishmeal with SCP concentration in diets of rainbow trout had resulted in marked reduction in growth performance and feed utilization. But partial replacements for fish meal in a complete ration had no marked adverse effects.

Table 2.20 Comparative values of amino acid from seven phototrophic bacteria and other commercial SCP (Vrati 1984)

Protein Source	Amino acid (as percent protein)							
	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenyalanine	Threonine	Valine
Rps. capsulata	2.82	5.24	8.02	5.41	3.23	5.23	5.12	7.20
Rps. palustris	2.02	4.32	7.23	5.20	3.33	4.22	4.86	6.50
Rps. acidophila	2.75	4.43	6.88	4.82	3.41	4.43	4.82	6.88
Rps. gelatinosa	3.02	3.98	7.01	4.66	2.88	4.80	4.75	6.44
Rps. sphaeroides	2.90	3.85	7.14	5.60	3.00	4.75	5.05	6.55
Rsp. rubrum	3.82	4.10	6.56	4.93	3.05	5.12	5.40	7.05
Rsp. tenue	2.80	4.30	7.72	5.05	3.41	5.20	4.80	7.30
Chlorella[a]	1.90	4.39	8.03	4.88	0.48	4.77	4.10	5.43
Yeast[a]	1.73	5.20	7.00	7.44	1.00	4.35	5.24	6.33
Meat[b]	1.8	3.4	6.4	5.0	1.3	3.6	3.4	5.0
Egg[c]	2.4	6.6	8.8	6.4	3.1	5.8	5.0	7.4
Soybean	2.4	5.4	7.7	6.3	1.3	4.9	3.9	5.2
Wheat flour	-	4.2	7.0	1.3	1.5	5.5	2.7	4.1

[b]Kobayashi & Kurata (1978); [d]Shipman et al. (1975); [c]Erdman et al. (1977)

Species of *Rhodobacter*, *Rhodospirillum*, *Rhodocyclus* and *Rhodopseudomonas* were appropriate for SCP, because they could grow both aerobically and/or photosynthetically utilized organic substrates (Sasaki et al. 1991). The protein content

of the bacterial cells (60-70%) was relatively high compared with that of yeast (50–60%). In most SCP manufacturing processes final cell densities were in order of 10-20 g dry cell/L. They also reported that the strain *Rhodocyclus gelatinous* was used not only to treat wastewaters but also to harvest bacterial cells as SCP.

SCP production using *Rhodobacter capsulata* from cow dung and swine waste have been possible. However, these wastes have to be anaerobically digested before use in SCP production (Sasaki et al. 1991). Some practical problems in SCP production in agro-industrial wastes (Sasaki et al. 1991) are (1) availability of sufficient quantities and concentrations of wastes; (2) difficult to sterilize the wastes prior to inoculation with phototrophic bacteria; (3) difficult to maintain the predominance of phototrophic bacteria throughout the culture and high inoculum density is required to achieve cultivation within a short period; (4) biomass harvesting is costly compared with harvesting of yeast cells; (5) research and development of suitable photobioreactors are necessary especially to enhance illumination, and to maintain homogeneous mixing and optimal temperature.

One of the major constrain in microbial biomass production is the difficulty in separation process. Harvesting techniques for phototrophic microbial biomass are expensive. The harvesting of cells by flocculation appears to be a more convenient process than other conventional methods. Pushparaj et al. (1993) suggested that synthetic cationic polymer "Praestol" was effective in flocculation of unicellular organisms. Polymer "Praestol" a derivative of acrylic acid and acrylamide are available in powder form in market as trade name "Tillmanns S.p.A". Concentration of 1mg/L of Praestol in culture was observed to separate 86% of *Rhodopseudomonas palustris*, 70% of *Tetraselmis* and *Spirulina*. The other advantage of such flocculation

is that pH modification is not needed and no inhibitory effect on growth of the culture has been observed. However, separation of PSB cell is a problem as flocculation ability of PSB is relatively slow and thus a high cost of separation process has been reported (Watanabe et al. 1998). They reported that *Rhodovulum* sp. PS88, had strong flocculation ability and may be suitable for treating polluted seawater discharged from fisheries industries. *Rhodovulum* sp. PS88 a strain isolated from sea mud was found to have self-flocculating properties. Watanabe et al. (1998) further concluded that oxygen concentration enhanced the flocculation ability and more than 3 mg/L dissolved oxygen was required for higher flocculation.

2.2.5 Uses of phototrophic bacteria in aquaculture

The nutritional value of phototrophic bacteria clearly indicated that it could be used as a potential protein supplement. Kobayashi & Kobayashi (2001) reported 96.5% survival rate of carp when PSB was fed at 0.1% supplemented with commercial feed. Later Kobayashi et al. (1978) reported that phototrophic bacterial cells that were used in the purification of wastewater utilized as food by plankton, fish, and could also be used as a feed for the cultivation of *Artemia salina* (brine shrimp).

There are advantages in using phototrophic bacterial cells incorporated in aquaculture diets. Norparatnaraporn & Nagai (1986) concluded that phototrophic bacterial cells incorporated in feed of juvenile fish have advantages, such as (1) prevent fish diseases; (2) accelerate fish growth; (3) improve quality of fish meat; (4) maintain water quality in ponds.

PSB cells have also been reported to contain anti-virus substances. Kobayashi & Kondo (1984) reported that the addition of *Rhodobacter capsulatus* bacterial biomass

is that pH modification is not needed and no inhibitory effect on growth of the culture has been observed. However, separation of PSB cell is a problem as flocculation ability of PSB is relatively slow and thus a high cost of separation process has been reported (Watanabe et al. 1998). They reported that *Rhodovulum* sp. PS88, had strong flocculation ability and may be suitable for treating polluted seawater discharged from fisheries industries. *Rhodovulum* sp. PS88 a strain isolated from sea mud was found to have self-flocculating properties. Watanabe et al. (1998) further concluded that oxygen concentration enhanced the flocculation ability and more than 3 mg/L dissolved oxygen was required for higher flocculation.

2.2.5 Uses of phototrophic bacteria in aquaculture

The nutritional value of phototrophic bacteria clearly indicated that it could be used as a potential protein supplement. Kobayashi & Kobayashi (2001) reported 96.5% survival rate of carp when PSB was fed at 0.1% supplemented with commercial feed. Later Kobayashi et al. (1978) reported that phototrophic bacterial cells that were used in the purification of wastewater utilized as food by plankton, fish, and could also be used as a feed for the cultivation of *Artemia salina* (brine shrimp).

There are advantages in using phototrophic bacterial cells incorporated in aquaculture diets. Norparatnaraporn & Nagai (1986) concluded that phototrophic bacterial cells incorporated in feed of juvenile fish have advantages, such as (1) prevent fish diseases; (2) accelerate fish growth; (3) improve quality of fish meat; (4) maintain water quality in ponds.

PSB cells have also been reported to contain anti-virus substances. Kobayashi & Kondo (1984) reported that the addition of *Rhodobacter capsulatus* bacterial biomass

to commercial feed suppressed gill corruption disease of lobster and Marek's disease of fishes (type of virus disease).

Hirotani et al. (1991) reported that phototrophic bacteria have some anti-virus compounds that suppress viral diseases of shrimp as well as shellfish. In Japan, gill disease of prawn was prevented completely by adding the anoxygenic phototrophic bacteria in the tank (Kobayashi & Kobayashi 2001).

Phototrophic bacterial biomass could also be used as feed supplement or added partially to various fish diets. Growth of tilapia, fancy carp, crucian carp and prawn increased when they were fed with diet mixed with phototrophic bacteria. Noparatnaraporn et al. (1987) used fresh phototrophic bacterial cells of *Rhodobacter sphaeroides*, grown in pineapple wastewater, as feed in culture of red tilapia and fancy carp. They also used *Rhodocyclus gelatinosus* cells grown in cassava waste for gold fish culture. They observed no difference in mortality between control and PSB-supplemented diets. The phototrophic bacterial biomass was readily consumed and no toxicity was observed. Fish fed with phototrophic bacteria mixed with commercial feed had significantly superior growth. Specific growth rate was observed to be 9.9% per day with control diet, whereas feed that contained PSB cell showed a specific growth rate of 11.69% per day. At the end of 122 days gold fish gained a weight of 99.1 mg/day with the control diet, but 116.79 mg/day when fed with phototrophic bacterial incorporated diet.

Burgess et al. (1993) added a fish growth hormone gene into *Rhodobacter* sp. strain NKPB0021. The bacterial biomass was used as a supplement into diet to accelerate fish growth. They observed that the growth hormone was required in small quantity to

promote fish growth. The bacteria containing active recombinant growth hormone can be easily grown in seawater and used directly as marine fish feed supplement.

Qiao et al. (1994) compared the effects of adding *Rhodobacter sphaeroides*, *Rhodobacter capsulatus* and *Rhodopseudomonas palustris* in prawn feed during grow-out culture. They reported that all three bacterial strains fed to shrimp had shown increased weight compared to control. The weight increases were: *Rb. sphaeroides* 118.7%, *Rb. capsulatus* 113.6% and *R. palustris* 105.7%. They also observed improved water quality when fed with phototrophic bacteria, as ammonical nitrogen was observed to be significantly lower with control diet.

Kobayashi & Kobayashi (1995) also reported that when live cells of phototrophic bacteria were added at 0.1% (w/w) to the formulated feed given to the fry of crucian carp soon after hatching, an increasing of 27.2% survival rate was obtained. Further, the mortality was minimum at 3.5%. During winter Kobayashi & Kobayashi (1995) observed that in grow out pond water quality deteriorated and consequently abnormal death of fry was observed. The water quality in which the pond was treated with phototrophic sulfur bacteria maintained providing a suitable environment for normal growth in fry.

Manju & Dhevendaran (1997) used *Rhodospirillum rubrun* with fish meal and fed it to juvenile fresh water prawn. They concluded that the highest growth was obtained with diet containing mixed bacterial strains (*Pseudomonas*, *Moraxella*, *Vibrio* and *Micrococcus*), but the food conversion efficiency in a mix diet of phototrophic bacteria was observed to be better. Manju & Dhevendaran (1997) concluded that among unconventional protein resources, single cell protein (SCP) of microbial origin could replace 25-50% of fish meal.

Phototrophic bacteria was not only used as feed supplement, but was also used as a complete diet in *Artemia* culture system. Getha et al. (1998) reported that locally isolated strain *Rhodopseudomonas palustris* strain B1 could be used as *Artemia* feed. The sago waste grown bacterial biomass contained low fat that would appear limited to be used as feed alone. They concluded that bacterial biomass could be a good supplement to diets rich in fat but poor in protein content. They observed better growth and survival of *Artemia* fed with a diet containing *Rhodopseudomonas palustris* and commercial *Spirulina*. Although, no significant differences were obtained in mortality rate among the diets composed with and without bacterial biomass. A significantly higher growth was observed with diet composed of bacterial biomass and *Spirulina*.

Banerjee et al. (2000) reported that phototrophic biomass strain PD1 grown in synthetic media (GMM) could be used as feed supplement in tilapia (*Oreochromis nilotica*) culture. The purple non-sulfur bacterium *Rhodovulum sulfidophilum* were combined with commercial tilapia feed. In 119 days of growth they obtained 14% higher survival and 30% higher body weight in tilapia fed with *Rv. sulfidophilum* biomass diet as compared to those without phototrophic bacterial biomass. They concluded that further research are required for determine the optimum level of bacterial biomass to be incorporated in diet, nutritional composition of the selected strain and a suitable and cheap substrate for producing nutritionally richer microbial biomass.

In addition to growth, gonadal development also increased with the addition of phototrophic bacteria in normal diets. Noparatnaraporn et al. (1986) reported that the ovary weight and number of eggs of gold fish increased due to vitamin E content in

phototrophic bacteria in the diet. The ovary weight and number of eggs in gold fish were 8.6% and 34.8% respectively when control diet used , but ovary weight and number of eggs increased to 41.2% and 68.5% respectively when fed with phototrophic bacterial mixed diet. The result had proven that the growth, gonad development and fecundity of the fishes were enhanced by the addition of phototrophic bacteria. Phototrophs contained vitamin-E, which enhanced gonad development in fishes (Sasaki et al. 1991)

Sasaki et al. (1991) mentioned that in some fish cultures in Japan, phototrophic bacterial cells have been widely used as the source of vitamins and trace minerals to cultivate healthy fish.

As phototrophic bacteria contained carotenoids, the skin pigmentation of culture organisms could be improved with the addition of bacterial biomass into the diets. Noparatnaraporn and Nagai (1986) showed that the color of chicken egg yolk and skin color of carp and shrimp when fed with the phototrophic bacteria biomass supplemented commercial feed had been enhanced.

Noparatnaraporn et al. (1987) reported that the effects of carotenoids produced in *Rhodobacter sphaeroides* on fancy carp were noticeable when feed pellets were substituted more than 6.8 times with bacterial biomass; the skin pigmentation was increased, particularly in shades of deep orange and red.

Pond ecosystem could be improved by adding phototrophic bacterial biomass. Phototrophic bacteria could convert sulfite to sulfate and have high sulfur tolerance capacity. Phototrophic bacteria also have a strong nitrogen fixing ability.

Sasikala & Ramana (1995) reported that anoxygenic phototrophic bacteria are important in aquaculture because of their photosynthetic capability, fixing atmospheric CO_2 and thus could be used as primary producers in the pond ecosystem. Bacteria could also enrich the pond with nitrogen, because they had capability to fix N_2 and convert various organic/inorganic sulfides in waste into useful SCP along. Hence, they simultaneously reduced in COD and BOD

Cui et al. (1997) conducted an experiment in seed production of *Penaeus chinensis* fed a with mixture of four strains of *Rhodopseudomonas* sp. They concluded that the pond transparency improved by 20-cm, the abundance of attached organisms reduced by 50%, while grazing ability of shrimp increased. The most striking fact was faster metamorphosis (one day earlier) than the pond supplied with normal diet.

2.3 The Brine Shrimp: *Artemia*

2.3.1. Characteristics of *Artemia*

The brine shrimp, *Artemia*, a branchiopod crustacean that is commonly and widely uses as a live feed for larviculture of fish and crustaceans. Its most unique characteristic is its ability to become dormant inside a cyst (Van Stappen 1996). The cyst has the ability to hatch within 24 h after immersion in 25-30 ppt artificial seawater. *Artemia* are distributed all over the world along the shorelines of hypersaline lakes, coastal lagoons and solar salt works scattered over five continents. Although, *Artemia* are distributed widely, large portion of the commercially produced cysts are harvested from the Great Salt Lake, USA.

Van Stappen (1996) mentioned that with the expansion of aquaculture production since the 1970s, the demand for *Artemia* cysts soon exceeded supply and prices rose exponentially, turning *Artemia* into a bottleneck for the expansion of the hatchery aquaculture of marine fishes and crustaceans. The price of *Artemia* cysts becomes expensive and this has proved to be an important obstacle to hatchery development, particularly in developing countries.

2.3.2. Larval development

Larval development of *Artemia* is generally similar to other crustaceans, but its larvae are hardy and metamorphose faster. Van Stappen (1996) reported that upon immersion in seawater, the biconcave-shape cysts hydrate, become spherical and the dormant embryo soon become activated. After 20-h the outer membrane of the cysts bursts and the free-swimming nauplii hatched out. *Artemia* larva grows and differentiates through 15 molts. The first larval stage, known as instar I (400 to 500μm in length) is brownish-orange in color and possesses a red naupliar eye and three pairs of appendages. Instar I larva does not take up food as its digestive system is not well developed; thus the larva completely depends on its yolk reserves. After about 8 h, the larva molts into the 2nd larval stage (instar II). At this stage the larva starts to eat small food particles ranging in size from 1-50μm, such as, algal cells, bacteria and detritus. Food particles are filtered by its 2nd antennae and ingested by a functional digestive tract. From the 10th instar stage, an important morphological as well as functional change takes place as the juvenile undergoes sexual differentiation. Adult *Artemia* has an elongated body, with two stalked complex eyes, a linear digestive tract, sensory antennules and 11 pairs of functional thoracopods. Van Stappen (1996) further reported *Artemia* could live for several months under optimal

conditions. It reaches to adulthood from nauplius within 8 days and reproduces at a rate of up to 300 nauplii or cysts every 4 days.

2.3.3. Feeding Behavior

Feeding in *Artemia* passes through some critical stages, although, *Artemia* is a hardy and environmentally tolerant crustacean. Jones et al. (1987) mentioned that *Artemia* possesses a complex of proteolytic enzymes, which adapt to artificial feed, although *Artemia* larvae switched from herbivory to omnivory. In *Artemia* larvae trypsin activity was observed to be less than in penaeid mysis larvae.

According to Intriago & Jones (1993) culture of *Artemia* on a diet consisting solely of bacteria could not be done well. On the other hand, Rosowski (1989) mentioned that bacteria used as food supplements to brine shrimp had been well documented. They also found that C16:In-7 and C18:In-7 are the major fatty acids present in the bacterial fed *Artemia*. They also claimed that C18: In-7 originated from the elongation of C16:In-7.

Van stappen, (1996) reported that *Artemia* is a non-selective filter feeder of organic detritus, microscopic algae as well as bacteria.

Together with nutritional quality other physiological and environmental factors influence the feeding in *Artemia*. Dhont & Lavens (1996) mentioned several factors that influence the feeding behavior of *Artemia*. Filtration rate, ingestion rate and assimilation, water quality, developmental stages and culture conditions are important amongst them. They also reported that mass culture of suitable algae for *Artemia* is not economically realistic. All species of unicellular algae are not suitable for

Artemia. For example, *Chlorella* and *Stichococcus* have thick cell walls that cannot be digested by *Artemia*. *Coccochloris* produces gelatinous substances that interfere with food uptake. Some dinoflagellates produce toxic substances. They also mentioned dried algae give satisfactory growth performance, but these feeds are expensive. Another problem associated with dried algae is the high fraction of water-soluble component, which cannot be ingested by brine shrimp but deteriorates the water quality in culture unit.

Dhont & Lavens (1996) suggested the use of bacteria and yeast (as alternatives to micro algae) as *Artemia* feed. The single cell proteins have certain advantages to be used as fish feed, such as (1) cell diameter smaller than 20μm; (2) complete nutritional composition; (3) cell wall prevents leakage of water-soluble nutrients and (4) some of products available at acceptable cost.

They mentioned that according to Coutteau et al. (1990) supplement of *Artemia* diet with live algae or removal of complex and thick cell wall by enzymatic treatment significantly improved assimilation rate and growth rate of brine shrimp. They further stated that soluble material is not taken up by *Artemia* and will be decomposed in the culture media by bacteria. This deteriorates the water quality and builds up the toxic substances like ionic ammonia. Dhont & Lavens (1996) also stated that *Artemia* has a higher grazing rate over microalgae. Therefore, algal concentration in the culture unit should be monitored several times a day and accordingly have to maintain retention time for feeding the *Artemia*. They further suggested that according to Lavens et al. (1986) optimal feed levels coincided with transparencies of 15–20 cm during the first week of culture and 20-25 cm on the following day.

2.3.4. Growth of *Artemia*

Culture media and supply of food in the system have significant effects on the growth and survival of *Artemia*. Enrichments of *Artemia* with enriched ingredients and inert material are very common and well documented. According to Sorgeloos et al. (1980) eicosapentaenoic acid (C20: 5n–3) and docosahexaenoic acid (C22: 6n–3), two highly unsaturated fatty acids, are not required by *Artemia*. They added *Artemia* requires linoleic (C18: 2n–6) and linolenic acid (C18: 3n–3), which occur naturally in food such as rice bran and soybean oil.

Rosowski (1989) reported that according to Johnson (1980) at an initial density of 1 nauplius/mL, the best diet was blue-green alga *Spirulina*, with a length growth of 4 mm obtained on the 7th day. Person–Le Ruyet (1976) recorded a growth of 3.75 mm in *Artemia* on the 6th day with stocking at 2 nauplius/mL (cited by Rosowski 1989). Yasuda & Taga (1980) recorded the highest final *Artemia* density with *Chlorella* in combination with bacteria (cited by Rosowski 1989).

Rosowski (1989) also reported that according to Douillet (1987) dried sieved (44μm) foods like *Spirulina*, yeast, defatted rice bran, soybean and lacto-serum are incomplete foods for *Artemia*. Mixed foods with bacteria provide best growth for copulating adults on the 9th day.

Landau & Sanchez (1991) reported *Artemia* are more tolerant of ammonia than most of other aquatic organisms. They are normally found in isolated hyper-saline environments where few other organisms are able to live. They reported pH 6.5 stressful for newly hatch brine shrimp. It is likely that ammonia plus elevated H^+ combine to give higher mortality rates at the lower pH. They concluded that *Artemia*

nauplii that hatched from decapsulated cysts have more energy reserves. The reserved energy could be used in osmoregulation. Thus, decapsulated cysts nauplii could withstand the stress of high ammonia.

Intriago & Jones (1993) concluded that *Artemia* could be successfully cultured from nauplii to pre-adult solely on bacterium *Flexibacter* strain Inp3. They observed the highest growth and survival for the bacterium strain, mixed with algae (*Rhodomonas*). The remarkable point is that the *Artemia* survival was not always related to the final *Artemia* biomass. Thus, survival is not the best parameter for evaluating diets, but the final biomass in term of growth. They also added that according to Nimura (1980), *Artemia* growth could be retarded if overfed, probably due to a decrease in assimilation efficiency. The results of Intriago & Jones (1993) showed that the bacteria contributed not only as a food for the *Artemia* but also assisted *Artemia* in the digestion of the algae.

In addition to culture media and feed, stocking density in culture media also improves the growth and survival of *Artemia*. Dhont & Lavens (1996) reported that *Artemia* could be cultured at high to very high densities without affecting survival (Table 2.21). Inoculation densities of up to 5,000 larvae/L for batch culture, 10,000 larvae/L for closed flow-through and 18,000 larvae/L for open flow-through can be maintained. They also mentioned that maximum density caused no real interference on feeding behavior, but seems to affect the ingestion rate and therefore growth. The growth of *Artemia* is even better in artificial seawater than natural seawater (Dhont & Lavens 1996). The pH tolerance for *Artemia* ranges from 6.5 to 8.0. The pH tends to decrease during the culture period as a result of denitrification process. They reported low oxygen of less than 2 mg/L limits the production of *Artemia* biomass. Oxygen

concentrations higher than 2.5 mg/L are generally recommended. Maintaining oxygen levels of higher than 5 mg/L will result in the production of pale animals (low in respiratory pigment: hemoglobin) and reduced individual dry weight, have less attractive to predators. They also pointed that small air bubbles may be trapped between the thorapods and skim off the *Artemia* too.

Table 2.21. Survival and growth of *Artemia* at different stocking densities. (Dhont & Lavens 1996)

Density (ind./mL)	Survival (%)			Growth (mm)		
	Day 7	Day 10	Day 14	Day 7	Day 10	Day 14
5	82	77	58	3.3	4.7	6.1
8	66	50	56	3.2	4.8	5.2
10	95	73	72	1.8	2.9	3.5
13	61	32	28	-	-	-
15	55	41	34	2.0	2.9	3.8
18	58	37	29	-	-	-
40	74	12	-	1.5	2.3	-

2.4 Prawn Larvae Culture

2.4.1 Importance of *Penaeus monodon*

The black tiger prawn (*Penaeus monodon*) is a commercially important species, commanding a high market value. Liao (1984) reported that among 24 cultured *Penaeus* species only eight species are cultured commercially on a large scale and *P. monodon* is the most profitable and available species in the Asian region. He further added that prawn research is lagging far behind that of fishes, but development of prawn industries is faster and progressing well.

Kongkeo (1991) reported that small backyard hatcheries of *Penaeus* in Thailand are expanding rapidly and contribute greatly to the success of the prawn aquaculture

industry. These backyard hatcheries use simple technology with low costs and are totally managed by local farmers. They have their own micro-algal system for mass culture.

2.4.2. Larval rearing

The larval rearing methods of *Penaeus monodon* depend on the geographical area, because methods have been developed on the basis of availability of local material, species, feed ingredients and management techniques. Liao (1984) mentioned that larval rearing techniques of *Penaeus japonicus* are well established because of the high tolerance of larvae to environmental factors. The rearing technique for *P. monodon* is considered more difficult than that of *P. japonicus*.

Penaeid larvae pass through certain critical stages, which must be given particular attention. Typical characteristics of hatching cycles in prawn are shown in Table 2.22. Appropriate sizes of quality feed, careful handling in nursing and maintenance of water quality are important factors in successful culture. Liao (1984) stated that in culture of *Penaeus japonicus*, in order to raise the desired number of post-larvae, the most important is to rear them successfully through the protozoeal stage. Larvae in the mysis stage are much stronger and could easily be kept alive for a long period. After mysis, the post larvae are even stronger. Therefore, the protozoeal stage of penaeid prawns is the most difficult and critical stage. He also stated that post larvae older than PL-5 does not need so much attention, because they actively start to search for larger food.

Supply of quality feeds in a particular hatchery is important as it affects larval development. Liao (1984) pointed out that there are no established standard methods

in larval rearing of *P. monodon*. As protozoeae are light sensitive, so darkroom type hatcheries have advantages, but in some hatcheries tanks are covered only during protozoeal stages. Thus, the range of light tolerance, water salinity and other environment parameters are important in larval rearing studies.

Table 2.22 Characteristics of hatching cycle of prawn (Hanson & Goodwin 1977)

Stage of larvae	Duration of stage (days)	Common food used	Size at end of stage (mm)
Egg	1	---	0.25
Larva			
Nauplii (stages I-V)	2		0.50
Protozoea (stages I-III)	4	phytoplankton (5-10μm in size)	2.2
Mysis (stages I-III)	3	Zooplankton, *Artemia*, yeast	5.0
Prawn			
Post-larva (suspended in water)	6	*Artemia*, pellet feed	7.5
Post-larva (bottom dwelling)	14	Marine life, benthic, pellet feed	20.0

Chwang et al. (1986) suggested that it was more convenient to monitored the light conditions and easier to maintained the water temperature in indoor type hatcheries. Under dim lighting, larvae distribute evenly throughout the water column. Feed distribution and feed consumption are then more efficient. They further suggest that larval rearing in enriched water method is best for mass production. In enriched water methods water is rarely exchanged and wastes deposited at the tank bottom are not disturbed. Thus, an ecological balance is maintained between larvae, algae, microorganisms and colloidal organic particles. The larvae in the rearing tank consume these organisms.

Liong (1995) reported that during the protozoeal stages there is little need to exchange the culture water, but these must be an adequate supply of microalgae. From the mysis

stage onwards partial water exchange is necessary to maintain suitable water quality. He further added that excessive use of artificial diet leads to fouling of water, so water exchange becomes essential.

2.4.3. Nutritional requirement and feeding

Availability of sufficient feed in the culture tank and acceptability of feed are one of the major factors, affecting successful larval rearing. The feed must have all the nutritional elements that the larvae instantly required at a particular stage. The newly developed digestive system may not be able to digest or absorb all the nutrients in the feed, even though it is nutritionally rich. Besides, the larvae take time to acclimatize to the new feed. Therefore, larval feeding behavior towards the new feed must be assessed and monitored carefully. However, peneaid larvae typically switched from being herbivore to omnivore during development.

Gopalakrishnan (1976) noted that differences in the biochemical content of ova may result in different nutritional requirements of larvae within a species. It is also true that chemical or nutritional compositions of algae are constant during exponential stage. He also added larval growth, particularly for *P. vannamei* and *P. stylirostris* are extremely dependent on diatoms both for metamorphosis and growth, whether *Artemia*, are provided or not. Finally he concluded that in larval feeding experiments percentage of survival or metamorphosis might not be the best method for testing the nutritional value of a diet if growth is not achieved.

Hanson & Goodwin (1977) pointed out that the protozoea does not swim well and must be kept suspended in water and fed by having its mouth collide with phytoplankton. *Chlorella* and *Skeletonema* are special algae for protozoae. Yeast can

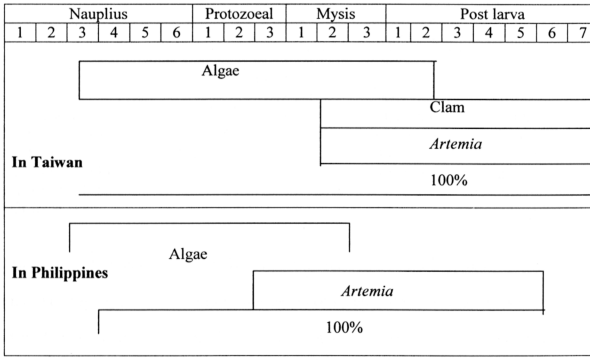
also be used as food. The protozoea swims constantly forward by action of the swimming legs and drag behind them a thread like feces. The mysis swims after its food and lives on zooplankton and other meat. Mysis could also feed on various forms of supplemental meat, namely grinded clams to be later displaced by addition of newly hatched *Artemia*. Mysis continue to feed on phytoplankton and will take microzooplankton e.g. rotifers and oyster eggs and larvae as well. It is clear to all aquaculturists that increasing demands for *Artemia* today are being accompanied by undeniably diminishing supplies and even higher prices.

Ward et al. (1979) stated that larval development is a critical stage in the life cycle of prawn in its natural environment. They added that any diet that is used to replace its natural food must contain the components required to ensure its normal development. They concluded that the major fatty acids of the eggs and nauplii are C16:0, C18:0, C18: 1, C20: 4 and C22: 6 which are required in larger quantities in postlarval stages than other fatty acids. They also mentioned that as larval development proceeds, the dependency on stored lipid decreases, and the prawn increases its dependency on external sources.

Jones et al. (1979) found 10 μm to be the optimum pellet size for Z_1 stage of *P. japonicus* fed a microencapsulated diet. Yang (1975, cited in Jones et al. 1979) reported that the Z_1 stage of *P. japonicus* was initially capable of ingesting food particles in the range of 3–5μm. At Z_3 stage the mouth of the larvae could take in cells larger than 200 μm. Jones et al. (1979) concluded that to ensure high growth and survival rates, feed must be of the correct size and the amount given should be carefully controlled. They reported that all larvae of *P. japonicus* fed at concentrations of 100 capsules/mL reached at the M1 stage within 10 days. At 1000 capsules/mL

level, larval mortality was high during the first 48 h and oversized capsules entangled the setose appendages of Z1 larvae. They suggested 500 capsules/mL is the preferable amount in *P. japonicus* larviculture system. Kurmaly et al. (1989) suggested protozoeal larvae of *Penaeus monodon* prefer to ingest spherical particles of up to 20μm diameter and in mysis stage up to 25μm. A schematic representation of general feeding regime of prawn larvae are shown in Table 2.23 and Table 2.24.

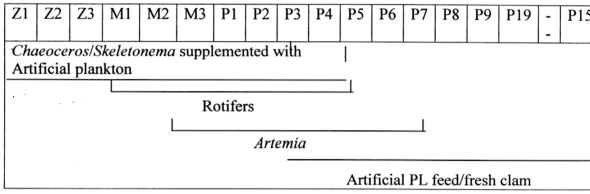
Table 2.23 Conventional hatchery feeding regimes for *Penaeus monodon* larvae. Solid (--) lines indicates live feed replaced by micro-encapsulated f eeds (after Jones et al. 1987)



Navarro et al. (1988) reported fish larvae that fed on *Artemia* nauplii lacking essential fatty acids showed typical ethological as well as morphological symptoms. In crustacean larvae lack of essential fatty acids causes abnormal metamorphosis, delay in molting processes and high mortalities. Navarro et al. (1988) also added there is a great lack of information on essential fatty acid requirements of early larval stages of

crustaceans in general and of penaeid larvae in particular. They concluded that metamorphosis could be the most sensitive point when judging and comparing the food value with different diets for crustacean larvae.

Table 2.24 Typical feeding schedule for *Penaeus monodon* larvae (after Kongkeo 1991)



	Amount of daily feed		
Feed	Protozoeal	Mysis	Postlarvae
Skeletonema	40,000/ml	50,000/ml	50,000/ml
or *Chaetoceros*	50,000/ml	60,000/ml	50,000/ml
Rotifers		100/larva	100/larva
Artemia nauplii	-	40/larva	100 – 200/larva
Artificial PL feed	-	-	20 – 50g/100.000
Or fresh clam	-	-	50 – 200g/100,000

Tacon (1990) mentioned generally four larval feeding strategies currently available for mass propagation of shrimp larvae, as (1) completely dependent upon use of live feed (*i.e.* algae, diatoms, flagellates, yeasts, rotifers, copepods, brine shrimp nauplii and meta-nauplii); (2) use of selected live and or frozen live feed together with fresh and / or frozen fish, mollusk or crustacean tissue preparations; (3) use of selected live and / or frozen live feed together with dry feed ingredients or formulated complete artificial diets and (4) total use of micro-encapsulated, micro-particulate or flaked artificial larval diets.

The best survival of larvae could be obtained if they were fed with nutritionally balanced feed. As larval life cycle passes through several stages, so nutrition requirements also vary with stages. The protein and lipid quality may be more important than amount of protein and lipid.

Kanazawa et al. (1985) mentioned that polyunsaturated fatty acids (PUFA) were found lacking in most of bacteria. Certain PUFA are essential in the diet of marine organisms. Eicosapentaenoic acid (20:6n-3), and docosahexaenoic acid (22:6n-3), are two highly unsaturated fatty acids (HUFA) essential for marine prawns.

Kanazawa et al. (1982) concluded that the protein requirement in penaeid larvae ranges from 45-50%. They reported that the diet must contain highly unsaturated fatty acids to ensure successful transition and survival of larvae. The availability of C20: 5ω3 (eicosapentaenoic acid) and C22: 6ω3 (docosahexaenoic acid) in the diet is very important. Although prawn larvae are able to elongate carbon chain C18: 3ω3 to C20: 5ω3 and C22: 6ω3, this reaction rate seems too slow to meet larval requirements.

Watanabe et al. (1983) reported that the essential fatty acids in live feeds are the principal factor in their dietary value.

Galgani et al. (1989) reported that larval rearing depends on a combination of feed uptake of the larvae and nutritional quality of food. They concluded that growth rate of *Penaeus monodon* larvae during protozoeal 1 to mysis 1 was apparently affected by increasing the concentration of *Tetraselmis*. They observed the highest percentage of successful molting at protozoeal substages (Z1, Z2 and Z3). The development from mysis to post-larval stages was not affected by food concentration. Finally they

concluded that to avoid food waste, underfeeding and water fouling, the particular larval stage where feeding response to a particular food is optimum must be known.

In aquatic ecosystem bacteria act as decomposers and utilizing decomposing organic materials to regenerate nutrients. According to Rieper (1978) bacterial role in aquatic system is not only regeneration and consumption of dissolved nutrients in the water column, but also serve as a primary food source for herbivorous zooplankton.

Lim (1991) mentioned that productions of live feed are most important operations in prawn and fish hatcheries. The live feed must be of the appropriate size, motile, palatable, and digestible and of high nutritional quality. In addition, the live feed must be hardy, able to reproduce rapidly and have the capacity to be mass produced under controlled conditions. He further reported that hatcheries in Singapore normally stock 100,000 *Penaeus monodon* nauplii/m^3 and one volume of diatoms is required daily for every 3.5 volumes of culture water.

Ouellet et al. (1992) stated that the successful larval development and metamorphosis depend on efficient utilization of energy reserves, especially lipids. The post-larvae could drive more energy from protein than from other nutrients, but for protozoea stage the main available energy is represented by lipids. They concluded that in penaeids naupliar stages depend largely upon energy reserves, primarily triacyl glycerol. They also concluded that insufficient energy due to low triacyl glycerol levels results in larval death at molt during the critical post larval stages.

Sorgeloos & Leger (1992) added that larval nutrition, particularly at first feeding by the early larval stage, appears to be the major bottleneck for the aquaculture industry. They also stated that feeding strategies used by hatcheries of marine fish and

crustaceans will probably never be standardized world-wide, because of species differences and geographical discrepancies. The cost-effectiveness of live and formulated feeds will dictate their proportional use. Coutteau et al. (1990) stated that because of its small particle size, high protein content and relatively low production costs, yeast has been considered as an algae substitute for several species of filter feeders. Nutritional value of any diet depends first on its degree of digestibility and second on its contents of essential elements. Yeast has a complex and thick cell envelope. Poor digestibility is the main constraint in its use of this single cell protein. Several methods could be used to improve the digestibility of SCP, e.g. mechanical disruption, autolysis and enzymatic treatment.

Kanazawa et al. (1982) mentioned that the optimum protein level in diets for penaeid prawn varied from 23% to 57%. The diversity is probably due to a variety of factors, such as differences in food habits, age of specimens, water temperature, sources of the protein used and energy level of the diet.

Reitan et al. (1993) stated that the algae in the larviculture system would supply various essential compounds (e.g. vitamins) in small amounts to the larvae, even though the biomass contribution often is low.

Rodriguez et al. (1994) stated that the ability of penaeid larvae to grow and survive on phytoplankton or zooplankton implies a high degree of flexibility in digestive physiology to fulfill nutritional requirements. According to Jones et al. (1993) despite the wide spread use of formulated feeds in penaeid larval culture, there is an urgent need for information on nutritional requirement of major dietary components especially protein, lipid and carbohydrates during larval life. Rodriguez et al. (1994)

claimed virtually nothing is known of the relative importance of phytoplankton and zooplankton in fulfilling such requirements.

Not all nutritionally qualified diets are digested 100% by peneaid larvae. With the development of stages, different types and amounts of digestive enzymes are secreted. Complete function of all enzymes are lacking in larval developmental stages. Galgani et al. (1985) concluded that penaeid larvae show higher enzymatic activity during herbivorous stages, but low enzyme activity during omnivorous stages. Normally the highest trypsin activity was observed between Z3 to M1 stages. This gives advantages for protozoeae to extract sufficient protein from diets to support metabolism and growth. Generally fish larvae required exogenous enzymes through live feed (Munilla–Moran et al. 1990, cited Jones et al. 1993), but Jones et al. 1993 concluded that contribution of exogenous enzymes from live feed is insignificant in penaeid mysis and *Macrobrachium* larval digestion systems.

Watanabe & Kiron (1994) reported that penaeid larvae initiate exogenous feeding and require extra energy to continue movement. When the larva turns to exogenous food, the amino acids necessary for energy must be provided by the ingested food. But it is yet not clear whether the larvae depend on free fatty acids by feeding (Fyhn 1989) or early proteolytic enzymes that are present in intestine (Ueberschar 1988, cited Watanabe & Kiron 1994). They concluded that in principle larviculture depends on live food. Although micro-feed taken over the places of live feed, but completely prepared food for larvae is still unknown.

Kumlu & Jones (1995) concluded that the fraction of algae, which stimulates trypsin enzyme activity, could be successfully incorporated in microencapsulated diet. The commercial availability of microcapsules containing algae extract should improve

survival and growth of herbivorous stages of penaeid larvae, thus, reducing dependency on live algae. They further added incorporate of predigested dietary ingredients might be necessary to overcome the poor digestibility. Jones et al. (1997) mentioned that most larvae of crustaceans are dependent upon enzymatic breakdown of ingested food. Among larval digestive enzymes proteolytic enzymes are found in all decapods larvae with trypsin as dominating enzyme. In *Penaeus monodon*, proteose, trypsin, pepsin, esterase, lipase and amylase are common that are active during larval stages. The mode of feeding of its larvae is from herbivory to omnivory and carnivory. They also observed the following percentages of enzymatic activity in larval stages of *Penaeus monodon*:

	Trypsin	Amylase	Lipase
Protozoel 1 to Mysis 1	95.2	2.8	2.0
Mysis 2 to Mysis 3	90.0	9.0	1.0
Post larvae 1	79.0	14.7	6.3

Sheen & Huang (1998) also stated that there was a relationship between enzymatic activity and larval survival. They observed the highest survival rate with high protease activity in penaeid larvae and low survival with low protease activity from protozoea to mysis. Protozoeae are herbivorous, but become carnivorous in the mysis stages during larval rearing. They concluded that larval diet should be designed according to the different feeding strategies. According to Rodriguez et al. (1994) differences in survival of larvae of *Penaeus japonicus* fed with *Chaetoceros gracilis* and *Artemia* nauplii lie in the digestive response of the larvae to the two feeds. Kumlu & Jones (1995) also indicated that the higher survival is due to higher digestive enzymatic activity in the larvae.

2.4.4. Types of feed

Studies have been done on the use of different types of live and artificial feeds to improve the survival, growth and metamorphosis of *Penaeus monodon* larvae (Liao 1984). With the development of larviculture, researchers are exploiting conventional and non-conventional feed resources for cheaper but quality feed. Chawang et al. (1986) suggested six to eight times of feeding per day. Live algal feed should be supplied throughout the larval period. From Z1 to Z2 stages, feed should be applied lightly, but in Z3 stage sufficient algae must be provided. Feed types also have effects on larval metamorphosis as shown in Table 2.25

Table 2.25 Duration of larval development and survival of *P. japonicus* fed with live feed and commercial micro-encapsulated diet (after Le Vay et al. 1993)

Diets used	Survival (%) to PL1	Duration (days)	
		To M1	To PL1
Live feed (*C. gracilis*, *Artemia* from mysis stage)	81.9	5	9
Microencapsulated Artificial diet (Frippak)	40.2	6	10.5
Frippak plus *C. gracilis*	75.9	5	9

Various types of feed uses in rearing penaeid prawn larvae and these are well documented. Table 2.26 also gives some food items used in penaeid larviculture.

2.4.5. Prawn growth based on artificial diets

Feeds for penaeid larvae cover a wide range of ingredients. Live feeds that are available for larvae, may not qualify as a nutritional requirement. The necessity of

artificial diets arises with the requirement of larvae in different stages. Penaeid larvae could be reared completely on an artificial diet (Bautista et al. 1991; Jones et al. 1993; Muir & Sutton 1994), but growth and survival seems satisfactory when mixed with micro-algae (Le Vay et al. 1993; Kumlu & Jones 1995). It has been proven that if the survival and growth are high at initial stages, a better growth and survival could be achieved in final in PL stages (Sheen & Huang 1998).

Table 2.26 Food items and feeding regimes for various developmental stages of penaeid prawns (after Liao 1984).

Food item	Zoea	Mysis	Postlarvae (early: P_1-P_{10})	References
Vegetable sources				
Skeletonema sp.	++	++		Hudinaga 1942
Tetraselmis sp.	+	+		Beard et al. 1977
Isochrysis sp.	+	+		Beard & Wickins 1980
Chaetoceros sp.	+	+		Hirata et al. 1975
Dunaliella sp.	-	-		SEAFDEC 1981
Spirulina sp.	-	-		Tang 1977
Chlamydomonas sp.	-	-		Hudinaga & Kittaka 1975
Marine *Chlorella*	-	-		Hudinaga and Kittaka 1975
Soy bean residue	+	+		Hirata et al. 1975
Animal sources				
Eggs or fertilized eggs of oyster	++	++		Liao 1969
Eggs of *Mytilus*	++	++		Kitaka 1975
Rotifer	++	++		Liao 1969
Artemia salina	++	++		Hudinaga 1969
Brine shrimp flakes	++	++		Unknown
Moina sp.			-	Kittaka 1975
Copepoda			++	Shigueno 1968
Gammarus sp.			-	Kittaka 1975
Balanus sp.			++	Kittaka 1975
Other sources				
Milled feed		+	+	Shigueno 1975
Sprayed dried feed		+	+	Shigueno 1975
Microencapsulated diet		+	+	Jones et al. 1979

Note: ++ Good; + Available; - Poor

Yashiro et al. (1985) reported that larvae of *Penaeus indicus* stocked at 100 nauplii/L and fed with micro-bounded artificial diet five times a day at 0.8 mg/larvae/day, had a survival rate of 45% from Z1 to M2 stage. They recorded water temperatures of 26.5-30.5 ^{0}C and salinities of 32-33-ppt. They found that the average survival rate of 70.2% from M1 to PL1 could be attained when stocking density was 30 nauplii/L; the larvae were fed three times a day at 0.3 mg/larvae/day, and water temperatures were 27-32 ^{0}C.

The major drawback in artificial particulate diet is nutrient leaching and thus fouling. Jones et al. (1987) pointed out that the use of microencapsulated artificial diet resulted in significant growth and survival of crustacean larvae. The artificial diet was assumed to be protects from dissolution and bacterial contamination. Recent artificial diet are produced as protein-walled microcapsules of different sizes, and could be dried, stored and re-hydrated for use as a larval feed. Jones et al. (1987) reared *Penaeus monodon* through all larval stages on a commercial scale in the absence of live feed. In addition to micro-encapsulated feed, yeast (*Sacharomyces cerevisiae*) was also added to all culture vessels to supplement the algae and *Artemia* diet from Z2 to M1 stages. They found that the larvae optimally ingested particles below 10 μm in size and use chemosensory selection when feeding on artificial particles.

Bautista et al. (1989) reported that kappa-carrageenan microbound diet (C-MBD) could be used as partial or total replacement for the traditional algal food (*Chaetoceros calcitrans*). The best larval development and the highest survival of 69.8% to PL were obtained with diet composed of C-MBD, *Chaetoceros calcitrans* and *Artemia salina*. Further, they stated that larvae, fed only with C-MBD had their

development from protozoeal to PL stages delayed by one day. However, total length that ranged from 3.6 to 3.8mm in PL stage did not showed significantly difference among diets. Larvae fed with natural algae and *Artemia* thus had faster metamorphosis and enhanced survival.

Liao et al. (1990) reported the usefulness of micro-bound diets for rearing larvae of *P. monodon*, where protozoea larvae could be reared up to the post larvae stages only with micro-bound diet. They obtained survival rate of 77% when larvae reared in a 0.5 ton tank were fed with micro-bound diet.

Tacon (1990) reported that live food organisms with inert or artificial diet resulted in reduced larval survival and delayed larval development. He further concluded that the causes of such results were (1) inadequate feed management techniques: frequent feeding and water exchange, (2) poor understanding of larval feeding behavior and feed requirement, (3) poor water stability of feed and (4) leaching of nutrients which increased water pollution.

Bautista et al. (1991) observed 42–56% survival in *P. monodon* larvae fed natural food mixed with a formulated diet. Metamorphosis was observed to be very slow up to PL1 stage. The larvae reached PL1 in 12–14th day. They did not mention what type of natural food they used. The larvae fed only with natural food attained 3% survival at PL1 stage. The formulated diet contained higher percents of protein and lipid compared to other diets. They further reported that the formulated diet contained high proportions of HUFA. The amount of fatty acid in other diet was comparatively lower which may not have been sufficient to allow the larvae to survive.

Sheen & Huang (1998) reported that the normal survival of penaeid prawn from protozoeal to post-larval stage was about 75-90% with micro-particulate diets containing Kappa carrageenan as a binder. More than 70% larval survival in *Peneaus monodon* could be obtained from nauplii to early juvenile stage using mciroparticulate diets. They mentioned that the protein quality and quantity of diets were not reflected in larval survival. The different protein source induces different responses in larval survival. They concluded that the protozoeal of *Penaeus monodon* should be fed with diets containing plant protein sources e.g. soybean meal, whereas in mysis stages *Penaeus monodon* should be fed with diets containing a combination of protein from animal sources.

2.4.6. Live feed for prawn larval rearing

Cultured live feeds are complements of natural diet. There are no alternatives of natural feed. During early development of larviculture, farmers had to depend on cultured live feed. Until now most of the larviculture techniques are based on live feed, though intensive research has been done in artificial diet. *Skeletonema* is one of the prominent diatom traditionally used in rearing prawn larvae during the protozoeae stage (Cook 1969). Culture of *Skeletonema* in control environment is easy, even in out door where it grows well.

Chwang et al. (1986) reported that in Taiwan the locally available diatom *Skeletonema* is widely used by hatchery farmers. Algae other than *Skeletonema* cannot be collected by 200 μm mesh nylon bag.

According to Kurmaly et al. (1989) *Skeletonema costatum* contains large quantities of ash due to its silica frustules. They reported the proximate composition of several marine phytoplankton among which *Skeletonema* possesses the lowest percentage of protein. They reported *Skeletomena* contained 33.3% protein, 8.1% lipid, 22.6% carbohydrates and 36% ash. The diatoms size ranged from 8-16μm.

Tacon (1990) pointed the disadvantages associated with an intensive live feed hatchery feeding strategy. He stated that prawn larval hatchery using a live feed could be economically managed. The major disadvantages associated with live feed culture are (1) high start up capital investment cost: unit for live feed production facilities including laboratory with high energy service requirements; (2) land/space requirement: requires extra space, as at least 20% of total larval rearing tank capacity is recommended for algal production (Kungvankij, 1982); (3) maintenance of stock culture: air conditioned laboratory required to maintain pure culture; (4) labor requirement: to maintain large-scale production of live feed, high labor input and skill technical manpower is required; (5) small scale or backyard hatchery development: small-scale farmers with limited cash funds and lack of technical expertise, do not produce enough live feed; (6) weather effects: out-door cultures completely depend on climatic conditions, thus the cycle of larval rearing are also affected; (7) variable quality and nutritive values: the quality and nutritive values of the live feed varies with strain, sources and culture methods; (8) availability and cost: the estimated cost of dry weight rotifers and *Artemia* nauplii were US$2000/kg and US$220/kg respectively, based on culture techniques at the Center Oceanologique de Bertagne, France (Girin, 1977) and (9) risk of larval infection: live food organisms may be hosts or carriers of pathogens that could be transferred to the developing larvae.

Kongkeo (1991) mentioned that within six hours after constant exposure to strong sunlight algae are ready for further sub-culture. Any delay in harvesting or subcultureing results in over-blooming and culture collapse within 4 to 6 h. *Skeletonema costatum* culture collapse is characterized by clumps of plasmolyzed cells, turbidity high and milky coloring of culture media. The main disadvantage of *S. costatum* is that after three to four generations the sizes reduced to 30–50% in length and width. So it becomes necessary to replenish the culture regularly with new strain. In general *Skeletonema* grew fast, easily harvested and could be available in natural seawater. Thus, many farmers prefer to use *Skeletonema* as initial live feed in penaeid larvae (Table 2.27).

Table 2.27 Assessment of two planktonic live feeds in penaeid larval rearing in Thailand (after Kongkeo 1991)

		Skeletonema	*Chaetoceros*
1.	Larval survival	60%	70%
2.	Nutrition value	Good	better
3.	Digestibility	Poor (long-chained, thick cell wall)	good (mainly single-celled, thin cell wall)
4.	Availability of natural stock	throughout the year	some seasons, low quantity
5.	Contamination	little	easy
6.	Mixed culture technique	possible	nearly impossible
7	Optimum temperature	26 –28° C	28 – 30°C
8.	Minimum temperature	20°C	24°C
9.	Optimum salinity	27 –30 ppt	22 – 28 ppt
10.	Light intensity requirement	< 10,000 lux	> 10, 000 lux
11.	Peak of growth	within 6 – 10 hours	within 12 – 24 hours
12.	Harvesting	easy (by filtration)	difficult(only transfer with culture water
13.	Cell degradation	within 3 – 4 generations	after more than 10 generations

Skeletonema culture is very simple and could be grown easily on synthetic seawater. The most common media used in the production of diatoms is TMRL media (Table 2.28).

Table 2.28 Modified TMRL medium for diatoms culture (after Kongkeo 1991)

Skeletonema costatum and *Chaetoceros calcitrans* (Modified TMRL medium)	
Urea	200g
Na_2HPO_4. $12H_20$	20g
$FeCl_3$. $6H_2O$	6g
$Na2SiO_3$ $9H_2O$	4g
Seawater	1ton

Lim (1991) described two staged culture techniques of *Skeletonema* in Singapore. At the first stage, *Skeletonema* stock was cultured outdoor in 0.5-1.0 m^3 glass fiber tanks with synthetic nutrient media. The compositions of the nutrients media (per m^3) are KNO_3 (300g), Na_2HPO_4 (30g), $FeCl_3$ (5g) and Na_2Sio_3 (15g). Lim (1991) further added diatoms inoculated at 5000-10000 cells/mL with strong aeration of 20 L/min. Within one-two days peak density was achieved at 40,000- 60,000 cells/mL. The culture was then ready to be transferred for mass cultivation. At the second stage, mass culture was conducted in 5-12 m^3 glass fiber tanks. In 8 m^3 capacity tank containing 3 m^3 of seawater, cells were inoculated at 5000-10,000 cells/mL. When density increased to 40,000 or more cells/mL another three parts of nutrients were added. At the following day the diatoms reached their peak density and color looked brown.

Jones et al. (1997) stated that biochemical composition of algae food depends on temporal and environmental factors. Absolute protein content of phytoplankton,

particularly siliceous diatoms might be lower than some of zooplankton. Since, it is common for all herbivorous feeders to have high ingestion rates but short gut retention times, an intake of up to eight times the larval body weight per day was proposed for penaeid protozoeae.

2.4.7. Growth of larvae fed live feed

Algae as live feed encouunted several difficulties. None of the single celled algae was found to be completely suitable for larval rearing up to PL stages. The algal foods are supportive with other artificial or encapsulated diets.

Ryther & Goldman (1975) stated several causes of unsuitability of algae as larval food. These are wrong sizes or shapes, indigestibility of thick cellulose, cell walls or deficiency in one or more essential nutrients, and release of toxic or inhibitory metabolites by algal cells. They also mentioned that most species of marine algae might produce biologically active (i.e. inhibitory) substances at certain stages of theirs life cycle.

Hanson & Goodwin (1977) added larvae could be raised up to PL-2 on *Skeletonoma* and *Tetraselmis* alone. Japanese hatcheries are now using less *Skeletonema* (Simon 1978). The reason is the problem of maintaining the algae with larval culture above 25 ^{O}C and the predominance of other diatoms.

Tobias–Quinitio & Villegas (1982) reported that the survival and growth rate of *P. monodon* larvae depend on the cell sizes of plankton. Cells of *Chaetoceros calcitrans* are smaller (4–5μm) than cell sizes of *Tetraselmis chuii* (12–15μm). Because of small cell sizes of *Chactoceros* the larvae of *P. monodon* ingested more cells, thus better

growth and survival were attained. Particle size is an important factor in larval rearing. They mentioned that it is not only protein quantity, but also the quality of protein that enhances growth and survival. They mentioned that proteins from two or more sources are better, because of improvement of the amino acid profile.

Aujero et al. (1985) concluded that *Peneaus monodon* larvae fed with combined diets gave survival rates comparable to or higher than those fed with algae or marine yeast alone. When used in combination with algae, nutritive values of the diet could be improved.

Kurmaly et al. (1989) mentioned that *Skeletonema costatum* could cause mechanical fouling. Cell aggregates on the protozoeal cephalic appendages perhaps inhibit effective feeding and respiration. They concluded that combination of algae and artificial diet might represent a more nutritionally balanced and a more digestible diet, and algae may additionally function as a biofilter removing toxic waste from the culture water. Kurmaly et al. (1989) further mentioned that the protein content of *Cheatoceros gracilis* (6.9% dry weight basis) is below that of *Skeletonema costatum*, *Tetraselmis chuii* and *Chaetoceros calcitrans*.

Kongkeo (1991) reported that prawn hatcheries in Thailand prefer to use *Chaetoceros calcitrans* as its nutritive value and digestibility seems to be better. He also pointed out that the local strain of *Skeletonema* often causes high mortality due to its poor digestibility. *Skeletonema costatum* used in Thailand are imported mainly from Japan or Taiwan.

Lim (1991) reported four suitable micro-algal species supporting two penaeid hatcheries in Singapore. He claimed *Tetraselmis tetrathele*, *Isochrysis galbana*, *Chaetoceros calcitrans* and *Skeletonema costatum* are most commonly used to rear

banana shrimp (*Penaeus merguiensis*) and the tiger prawn (*Penaeus monodon*). *Skeletonema costatum* is widely used for the following reasons:

(i) its availability around the Singaporean coast throughout the entire year

(ii) does not have any serious contamination problem under local condition

(iii) can easily be harvested with 40 μm mesh plankton net.

Amjad et al. (1992) compared growth and survival of *P. monodon* larvae fed with micro-encapsulated feed and with a supplement of active growth factors taken from live feeds. The live feeds were mixture of micro algae *Tetraselmis chuii* and *Rhodomonas baltica* at a ratio of 1:1. They reported that larval fed with micro-encapsulated diet was not significantly different in growth and survival from larvae fed the control diet (algae and *Artemia*). They observed 93% survival in control diet. They also observed higher variations in growth when fed with encapsulated diet, because the encapsulated feed was not uniformly distributed in the larval culture system. They concluded that microencapsulated diet must be supplemented with other additives to achieve good growth and survival.

Malaysian hatcheries are not exceptions in using common diatoms for rearing peneaid prawn larvae. Liong (1995) reported that the most common microalgae used as first larval feed in Malaysia are *Skeletonema*, *Chaetoceros*, *Tetraselmis* and *Isochrysis*. All the prawn hatcheries have special facilities for large scale production of these selected microalgae. He also stated that formulated larval diet and dry algal preparations are also widely used, but these cannot completely replaced live food organisms in

hatchery operation. He further added that some prawn hatchery operators completely depend on live food alone and prefer not to use any artificial feed.

2.4.8 Water quality on larval rearing

Feed uptake and digestion are closely related to physiological and metabolic activities of penaeid larvae, which are governed by temperature and other environmental parameters.

Temperatures are crucial in feed uptake and metamorphosis of penaeid larvae. Cook (1969) mentioned that larval mortality varied with the initial stocking rate and availability of feed. Water temperature should be kept above 24 °C, but optimum temperature lies in the range of 28-30 °C. Besides adequate nutrition, the temperature of the rearing water is an important factor in metamorphosis of penaeid prawn and the optimum range of temperature is 28–32°C (Liong 1995). Temperature affects the time of molting from nauplius to zoea. Rees et al. (1994) mentioned that at 33 °C and 33 ppt salinity protozoeae could reach to mysis stages faster than at the temperature of 28 °C and 33 ppt salinity. The rate of metamorphosis in the outdoor tank is attributed to higher water temperature (31-32 °C), while indoor temperatures are within 26-27 °C (Kungvankij et al. 1986). Water quality parameters of open and closed larviculture system are shown in Table 2.29. Kungvankij et al. (1986) also mentioned that dry season temperature was suitable to raise *Penaeus monodon* larvae without any temperature control. Further, concluded that in outdoor tanks *Penaeus monodon* can reached PL stage on the 9th day, but in indoor tanks it took 11-12 days. Partial breakdown of food material together with excretion products increases the nitrogenous substances in culture system. This has caused lethal effects on the

penaeid larvae. Feeding ceased with high level of nitrogenous substances, thus reducing larval survival and growth (Chin & Chen 1987).

Table 2.29 Water quality parameters in different systems for larviculture of *Penaeus merguiensis* (Menasveta et al. 1990)

Water system	Temperature (^{0}C)	Salinity (ppt)	Dissolved Oxygen (mg/L)	pH	NH_4-N (mg/L)
Closed Recirculating System	23.0-28.0	33.0-35.0	5.50-8.87	7.00-8.87	0.001-0.006
Open	26.0-28.5	30.0-35.0	7.30-8.70	8.51-9.19	0.007-1.27

Ammonia and its intermediate product (nitrite) are common toxic to fish and crustaceans. In closed culturing system, the accumulation of ammonia and nitrite may have detrimental effects on prawns and their larvae (Chin & Chen 1987). Chen et al. (1990) observed that ammonia could increase to more than 0.8mg/L during the larval development even with frequent water replacement. Chin & Chen (1987) reported a safe level of 1.15mg/L ammonia–N for the rearing of larval *P. monodon*. They further added that the nauplii and mysis stages of *P. monodon* larvae were less tolerant to nitrite than to ammonia.

Menasveta et al. (1990) also mentioned that nitrogenous compounds are good indicators of water quality. Normal seawater ranges of these compounds are

0.02-0.04 mg/L NH_4-N,
0.01-0.04 mg/L NO_2 –N and
0.1-0.20 mg/L NO_3-N

Uses of excess live feed, artificial particulate or encapsulated diet causes fouling in culture unit. These nutrient rich feeds encourage pathogenic bacteria to grow. Use of natural food for prawn larvae increases the possibility of water contamination by

exogenous pathogens (Liao et al. 1990). To avoid such circumstances optimum feeding, frequency, frequent exchange of water and double filtered recycled water are recommended. Water re-circulation systems (closed system) are alternative solution for larviculture of penaeid shrimp, where water quality is limited by turbidity, fluctuating salinity or eutrophication (Menasveta et al. 1990). They further mentioned to maintain good water quality in good condition used water should be filtered efficiently. The breakdown and leaching of unstable diets rapidly led to bacterial growth and accumulation of NH_4–N, NH_3-N and NO_2-N (Amjad et al. 1992). They concluded despite water exchange of 50% per day, ammonia and nitrite levels did not decline. Exchange of water is necessary to maintain the range of pH (Chwang et al. 1986). Further they added the pH values in larval rearing tank must be kept steady between 7.7 and 8.3.

2.4.9. Economic assessment of live feed

Live feeds, such as algae and copepods used in penaeid larviculture are readily available in coastal areas. The culture technique is also simple, but laborious and labor intensive. Even though the economic viability depends not only on labor factor, but long-term benefit should be taken in to consideration.

Hirata et al. (1975) reported that production of diatoms and their use in the frozen, freeze-dried and live form requires manpower and specialized equipment for cultivation and concentration of the algae. To avoid these problem Hirata et al. (1975) suggested that protozoeae be reared with detritus as food, but the problem is how to produce good detritus, as the process is expensive. According to Liao (1984) it is better to follow the principle of natural selection for greater economic utilization. The

use of marine yeast in larval rearing could lower economic and technological inputs in the production of natural foods (Aujero et al. 1985). Aujero et al. (1985) further stated that live feed could be grown on locally available cheaper carbon sources like molasses, brown sugar and coconut water with added nutrients in a relatively shorter period of time.

Kuban et al. (1985) concluded that a diet supporting only survival and metamorphosis was not appropriate if growth in biomass not achieved, as larger 1-day old postlarvae had a significant economic advantage in the grow-out industry. The diet must be attractive, ingestible and provided adequate vitamins and minerals for metabolites (Kurmaly et al. 1989). Liao et al. (1990) suggested that nutritionally rich micro-bound diets are environmentally suitable for raising *Penaeus monodon* larvae. They suggested that spray-drying methods are economical in micro-bound diet techniques than freeze-dried types.

Sorgeloos & Leger (1992) mentioned that understanding of microbial environment in the hatchery as well as larval immune system is necessary for a better economic output product.